UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

ANA ELIZABETH CAVALCANTE FAI

PRODUÇÃO ENZIMÁTICA DE GALACTOOLIGOSSACARÍDEOS, A PARTIR DA LACTOSE, POR *Pseudozyma tsukubaensis, Pichia kluyveri* e *Aspergillus oryzae.*

TESE DE DOUTORADO APRESENTADA À FACULDADE DE ENGENHARIA DE ALIMENTOS UNICAMP PARA OBTENÇÃO DO TÍTULO DE DOUTOR EM CIÊNCIAS DE ALIMENTOS

Orientadora: Dra. Gláucia Maria Pastore

Este exemplar corresponde à versão final da tese defendida por Ana Elizabeth Cavalcante Fai, aprovada pela comissão julgadora em 16/12/2011 e orientada pela Profa. Dra. Gláucia Maria Pastore.

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Ana Paula Manera
Janaína Nicanuzia dos Prazeres
Mário Roberto Maróstica Júnior
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Este exemplar corresponde à redação final da tese defendida por Ana Elizabeth Cavalcante Fai, aprovada pela comissão julgadora em 16 de dezembro de 2011.

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Prof[a]. Dr[a]. Gláucia Maria Pastore - Orientadora
FEA/UNICAMP

_____

Dr[a]. Janaína Nicanuzia dos Prazeres – Membro Titular
IQ/USP

_____

Prof. Dr. Mário Roberto Maróstica Júnior – Membro Titular
FEA/UNICAMP

_____

Dr[a]. Patrícia Blumer Zacarchenco Rodrigues de Sá – Membro Titular
TECNOLAT/ITAL

_____

Dr[a]. Rosângela dos Santos – Membro Titular
FEA/UNICAMP

_____

Prof[a]. Dr[a]. Ana Paula Manera – Membro Suplente
UNIPAMPA

_____

Dr. Haroldo Kawaguti – Membro Suplente
Quantas Biotecnologia S/A

_____

Prof. Dr. Yong Kun Park – Membro Suplente
FEA/UNICAMP

"Há um tempo em que é preciso abandonar as roupas usadas que já têm a forma do nosso corpo, e esquecer os nossos caminhos, que nos levam sempre aos mesmos lugares. É o tempo da travessia: se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos".

(Fernando Pessoa)

Ao meu esposo Pedro Henrique
Aos meus pais Graça e Andres (in memoriam)
Aos meus irmãos Maria Julieta e Rafael José
Aos meus avós paternos e maternos

Dedico esta tese

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RESUMO

Galactooligossacarídeos (GOS) são prebióticos sintetizados via transgalactosilação enzimática da lactose e existem vários modos de se obter os produtos sintetizados nesta reação. Com o objetivo de se produzir GOS e hidrolisar lactose, β-galactosidase de *Aspergillus oryzae* (Sigma®) foi covalentemente imobilizada em celite e em quitosana em pó ativada com glutaraldeído, sendo as propriedades destas avaliadas e comparadas em relação à enzima livre. Uma coluna de leito empacotado foi utilizada para a produção de GOS e hidrólise da lactose nos sistemas imobilizados. Os oligossacarídeos foram obtidos com um máximo de produtividade de 14,42 e 3,50 g/L.h, partindo de uma solução de lactose 40% (p/v), na coluna de leito empacotado com quitosana e celite, respectivamente. A hidrólise da lactose foi de 50,00 e 84,74% após 24 horas utilizando quitosana e celite como suportes enzimáticos, respectivamente. A fim de verificar a habilidade de síntese de GOS por *Pichia kluyveri* e *Pseudozyma tsukubaensis*, isoladas de pêssego (*Prunus persica*) e nectarina (*Prunus persica* var. *nucipersica*), respectivamente, utilizou-se células íntegras e viáveis para fermentar um meio de lactose a 40% (p/v). Rendimento máximo de 14,01 e 15,71% (p/p) de GOS foi obtido desta fermentação em 24 horas por *P. kluyveri* e *P. tsukubaensis*, respectivamente. Uma estratégia sequecial de dois planejamentos experimentais foi utilizada para otimização de rendimento de GOS e, sob as condições validadas, a atividade de transgalactosilação de *P. tsukubaensis* resultou em 28,35% (p/p) de rendimento de GOS em 24 horas, com uma produção de 73,71 g/L e aproximadamente 50% de hidrólise de lactose, a partir de uma concentração inicial de lactose de 26% (p/v).

Foi proposto, ainda, um processo associado de biossurfactante/biomassa para síntese de GOS a partir da lactose. O biossurfactante sintetizado por *P. tsukubaensis* em manipueira foi otimizado utilizando planejamento experimental e análise de superfície de resposta, considerando como resposta a tensão superficial e a concentração de biomassa. A mínima tensão superficial e a máxima concentração de biomassa observada foram de 26,87 mN/m e 10,50 g/L, respectivamente. A atividade de trasgalactosilação da biomassa de *P. tsukubaensis*, nas condições otimizadas do processo, resultou em uma produção de 73,12 g/L e em rendimento de 18,28% (p/p) em 24 horas, a partir de 40% (p/v) de lactose.

SUMMARY

Galactooligosaccharides (GOS) are prebiotics synthesized by enzymatic lactose transgalactosylation and there are many modes in which GOS producing reactions can be performed. In order to produce GOS and to hidrolise lactose *Aspergillus oryzae* β-galactosidase (Sigma®) was covalently immobilized on celite and on glutaraldehyde-treated chitosan powder and its properties were evaluated and compared with those of free enzyme. A packed bed column with lactose recycle was employed for production of GOS and lactose hydrolysis by immobilized systems. Oligosaccharides were obtained with a maximum productivity of 14.42 and 3.50 g/L.h, from 40% (w/v) lactose solution, in packed bed column with enzyme on chitosan and celite, respectively. Lactose was 50.00 and 84.74% hydrolyzed after 24 hours using chitosan and celite as enzyme support, respectively. In order to verify the ability to synthesize GOS from *Pichia kluyveri*, isolated from peach (*Prunus persica*), and *Pseudozyma tsukubaensis*, isolated from nectarine (Prunus persica var. Nucipersica), living whole cells were used to ferment 40% (w/v) lactose solution. A maximum yield of 14.01 and 15.71% (w/w) GOS was obtained from the fermentation in 24 hours by *P. kluyveri* and *P. tsukubaensis*, respectively. A sequential strategy of two experimental designs was used to optimize GOS yield and under the validated conditions the transgalactosylation activity of *P. tsukubaensis* resulted in 28.35% (w/w) of GOS yield at 24h, with a production of 73.71 g/L and approximately 50% of lactose hydrolysis, from 26% (w/v) initial lactose concentration. An associated process for biosurfactant/biomass for GOS synthesis from lactose was subsequently proposed.

Biosurfactant synthesis by *P. tsukubaensis* on cassava wastewater was optimized using experimental design on the response of surface tension and biomass concentration. The minimum surface tension and maximum biomass concentration predicted and experimentally confirmed were 26.87 mN/m and 10.50 g/L, respectively. The transgalactosylation activity of *P. tsukubaensis* biomass at optimized conditions from 40% (w/v) lactose resulted in a GOS production of 73.12 g/L and a yield of 18.28% (w/w) in 24 hours.

INTRODUÇÃO

As recentes comprovações científicas estreitando a relação entre dieta e saúde, e a valorização do consumidor por alimentos saudáveis trouxeram um novo desafio para a indústria de alimentos: o de se adaptar à era da nutrição otimizada (GOSLIN *et al*., 2010; OTIENO, 2010).

Dentre vários alimentos que encerram em si moléculas biologicamente ativas destacam-se os oligossacarídeos com efeito prebióticos, entre estes, os galactooligossacarídeos (GOS) (LAPARRA & SANZ; RAGHUVEER, 2009; ALMEIDA & PASTORE, 2004).

Além do efeito bifidogênico (KAUR & GUPTA, 2002; ROBERFROID, 2007) outros benefícios associados ao consumo de GOS são o estímulo do sistema imunológico do hospedeiro, redução nos níveis de bactérias patogênicas no intestino, alívio da constipação e diminuição do risco de câncer de cólon e osteoporose. Adicionalmente, estima-se que haja uma redução do risco de arteriosclerose, através da diminuição na síntese de triglicérides e ácidos graxos no fígado e diminuição do nível desses compostos no sangue (LIM, FERGUSON & TANNOCK, 2005; KAUR & GUPTA, 2002).

Quimicamente os GOS são formados por moléculas de galactose ligadas à glicose, sendo formados de tri a decassacarídeos com 2 a 9 unidades de galactose, respectivamente (LI *et al*., 2008). São produzidos a partir de soluções com altas concentrações de lactose, por atividade de transgaloctosilação, pela β-galactosidase (EC3.2.1.23), a qual pode ser extraída de diversas fontes, sendo a microbiana a mais usual (PARK & OH, 2010).

A enzima β-galactosidase é comercialmente importante, uma vez que, além da propriedade de produzir GOS, catalisa a hidrólise da lactose, sanando assim problemas associados com o descarte do soro, cristalização deste dissacarídeo em alimentos congelados e o consumo de leite por indivíduos com intolerância à lactose (GROSOVÁ, ROSENBER & REBROS, 2008).

Para a síntese de GOS pode-se utilizar células viáveis, a partir da fermentação do meio, bem como fazer uso de β-galactosidase extraída previamente, sendo, neste caso, uma das formas interessantes de processo a imobilização do biocatalizador.

Enzimas ou células imobilizadas são catalisadores fisicamente confinados ou localizados em uma região definida do espaço, com retenção de suas atividades catalíticas, e que podem ser utilizados repetida ou continuamente (HANELFELD, GARDOSSI & MAGNER, 2009; CHIBATA, 1978).

A opção de imobilização é principalmente induzida pela facilidade de controle da reação e possibilidade de reutilização do sistema, além de aumentar a estabilidade do biocatalizador em questão e permitir o escalonamento industrial (NERI *et al* 2009; GROSOVÁ, ROSENBER & REBROS, 2008).

Um dos reflexos da importância industrial do desenvolvimento de novos processos biotecnológicos de síntese de GOS é o expressivo número de pesquisas científicas desenvolvidas em torno deste tema nos últimos anos. Observa-se na literatura estudos para a produção deste açúcar funcional sob diferentes condições experimentais, visando encontrar um sistema com alta taxa de conversão de lactose em GOS e economicamente viável (PARK & OH, 2010; LI *et al*., 2008; SANTOS, SIMIQUELI & PASTORE, 2009; GROSOVÁ, ROSENBER & REBROS, 2008).

A importância e interesse em se buscar formas eficientes de produção de GOS, bem como de outros oligossacarídeos com características prebióticas, aumentam à medida que a compreensão de suas propriedades funcionais vão sendo deslocadas para diante (GOSLIN *et al*., 2010; BARRETEAU, DELATTRE & MICHUD, 2006; RASTALL & MAITIN, 2002).

A projeção para o segmento de ingredientes prebióticos para o ano de 2015, elaborada pela *Global Industry Analysts*, na Europa e nos Estados Unidos é de aproximadamente US$ 1,2 bilhão e US$ 225 milhões, respectivamente (NUTRACEUTICALS WORLD, 2010).

Assim, buscando encontrar soluções para o desafio posto, e considerando a grande biodiversidade brasileira, este projeto abrange dois aspectos de relevância: otimizar e comparar distintos processos para a síntese microbiana de GOS e pesquisar novos micro-organismos endofíticos potencialmente capazes de produzir este oligossacarídeo.

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OBJETIVOS

Geral

Síntese de galactooligossacarídeos, a partir da lactose, por micro-organismos pré-selecionados e por β-galactosidase comercial de *Aspergillus oryzae*.

Específicos

- Imobilização de β-galactosidase de *Aspergillus oryzae* (Sigma®) em quitosana e estudo da síntese de galactooligossacarídeos e hidrólise da lactose, bem como a averiguação das condições ótimas do processo.

- Imobilização da enzima β-galactosidase de *Aspergillus oryzae* (Sigma®) em celite, bem como a avaliação das melhores condições de atuação do derivado imobilizado para a produção de galactooligossacarídeos e hidrólise da lactose.

- Isolamento de micro-organismos endofíticos de frutas com potencial de transgalactosilação da lactose para a síntese de galactooligossacarídeos.

- Avaliação dos parâmetros estatisticamente siginificativos para a produção de galactooligossacarídeos, produzido pelos micro-organismos selecionados, e otimização do processo utilizando metodologia de superfície de resposta.

- Estudo da síntese de biossurfactante por *Pseudozyma tsukubanesis*, utilizando manipueira como substrato, com produção associada de galactooligossacarídeos.

CAPÍTULO 1

Revisão bibliográfica

GALACTOOLIGOSSACARÍDEOS: PRODUÇÃO, APLICAÇÃO EM ALIMENTOS E PERSPECTIVAS

GALACTOOLIGOSSACARÍDEOS: PRODUÇÃO, APLICAÇÃO EM ALIMENTOS E PERSPECTIVAS

Resumo

Sintetizados a partir da transgalactosilação da lactose, os galactooligossacarídeos são carboidratos não digeríveis, sendo considerados ingredientes prebióticos de alto valor agregado. Estudos recentes atribuem a estes oligossacarídeos uma série de potenciais benefícios a saúde e de prevenção de doenças. Esta revisão envolve os aspectos de produção destes compostos e aborda as suas propriedades físico-químicas, relacionando-as com seus efeitos fisiológicos e a aplicação na indústria de alimentos, buscando descrever as perspectivas vislumbradas para estes açúcares não convencionais, a partir do panorama atual.

1. Introdução

A correlação entre alimentos e saúde é inequívoca e atualmente os alimentos funcionais constituem uma importante área de pesquisa em todo o mundo (TURGEON & RIOUX, 2011; GRANATO, *et al*., 2010; DIAS, 2002; VERSCHUREN, 2002).

O apelo por produtos com características de qualidade que aportem ganhos fisiológicos aos consumidores, à parte das exigidas vantagens nutricionais, é a nova fronteira de expansão da indústria de alimentos (MARTINS & BUKERT, 2009).

Um alimento pode ser considerado funcional se for satisfatoriamente demonstrado que este apresenta um efeito benéfico em uma ou mais funções fisiológicas alvo, além do valor nutricional inerente à sua composição química, relevante para melhorar, manter e reforçar a saúde (BLUNDELL, J., 2010; LAPARRA & SANZ, 2010).

Atualmente a legislação brasileira, pela Resolução nº 18 (BRASIL, 1999) destaca que as alegações de propriedade functional podem fazer referências à manutenção geral da saúde, ao papel fisiológico dos nutrientes e não nutrientes e à redução de risco a doenças. Não são permitidas alegações de saúde que façam referência à cura de doenças. Ressalta-se que um alimento funcional deve configurar-se como um alimento e os seus efeitos devem ser demonstrados em doses que possam ser normalmente consumidas em uma dieta.

Nesta perspectiva, os alimentos funcionais e especialmente os oligossacarídeos com efeito prebiótico são conceitos novos e estimulantes (DELGADO, TAMASHIRO & PASTORE, 2010; ROBERFROID, 2002).

Os oligossacarídeos são açúcares encontrados como componentes naturais em muitos alimentos como frutas, vegetais, leite e mel, podendo ser sintetizados enzimaticamente por várias espécies de fungos, leveduras e bactérias (BIESIEKIERSKI *et al*., 2011; HSU, LEE & CHOU, 2007; CHOUCARABIN & FLAMM, 1999).

Segundo Fooks & Gibson (2002), os oligossacarídeos com características prebióticas estão entre as fibras que proporcionam efeito positivo na composição da microbiota intestinal quando consumidos, sendo resistentes às ações das enzimas salivares e intestinais.

Estudos recentes associam o consumo de prebióticos à modulação de funções fisiológicas e processos bioquímicos chaves, proporcionando benefícios a saúde e redução do risco de aparecimento de diversas doenças (RASTALL, 2009).

Entre os prebióticos, destacam-se os galactooligossacarídeos (GOS) os quais são produzidos a partir de soluções com altas concentrações de lactose por atividade de transgalactosilação, pela β-galactosidase, previamente extraída de diversas fontes, sendo a microbiana a mais usual (PARK & OH, 2010), por fermentação da lactose (ROY,

DAOUDI & AZAOLA, 2002; ONISHI & TANAKA, 1998), entre outros processos biotecnológicos afins (MANERA *et al*., 2011; PANESAR, 2008).

Convém mencionar que outro tipo de oligossacarídeo não transgalactosilado e não digerível também pode ser formado a partir da lactose por síntese química: a lactulose é um dissacarídeo composto por uma molécula de galactose e outra de frutose, unidas por ligação do tipo β-1,4 e resulta de um processo álcali de isomerização da lactose, na qual a galactose é isomerizada a um resíduo de frutose (BUHRING, FISCHER & HINRICHS, 2010; OLANO & CORZO, 2009). Os α-galactooligossacarídeos, por sua vez, são obtidos naturalmente da soja, incluindo a rafinose, estaquiose, melibiose e verbascose, os quais consistem em resíduos de galactose ligados a moléculas de sacarose por ligações do tipo α-1,6 e tampouco são digeríveis, devido a ausência de α-galactosidase entre as enzimas digestivas, sendo fermentados pela microflora gastrointestinal (TUNGLAND & MEYER, 2002). Esta revisão visa abordar somente os GOS obtidos via trasgalactosilação por ação da β-galactosidase.

Os GOS são caracterizados como ingredientes seguros para o consumo ou GRAS (Generally Recognized as Safe), uma vez que são componentes do leite humano e tradicionalmente de iogurtes, sendo ainda produzidos no intestino por micro-organismos produtores de β-galactosidase a partir da lactose ingerida. São naturalmente encontrados na soja e não apresentam toxicidade, somente diarréia é relatada como efeito adverso quando este açúcar não convencional é consumido em excesso (OTIENO, 2010; SAKO, MATSUMOTO & TANAKA, 1999).

Muitos são os efeitos benéficos dos GOS estimados na literatura e estudos envolvendo a síntese por diferentes biocatalisadores e a otimização da produção destes compostos, revestem-se de importância uma vez que envolvem aspectos de relevância

atual, como a produção de açúcares funcionais e a diversificação de produtos alimentícios (TORRES *et al*., 2010; TUOHY *et al*., 2003).

Neste contexto, esta revisão bibliográfica contempla os aspectos relacionados à produção microbiana de GOS, bem como os benefícios à saúde creditados ao consumo destes prebióticos. Aponta-se ainda algumas aplicações dos GOS na indústria de alimentos e quais são as perspectivas de mercado para estes ingredientes funcionais nos próximos anos.

2. Produção

Os GOS são formados a partir de substratos ricos em lactose via transgalactosilação, e para tanto podem-se utilizar células viáveis a partir da fermentação de substratos ricos em lactose, bem como fazer uso de β-galactosidase extraída previamente, sendo uma das formas interessantes de processo a imobilização do biocatalizador (BICAS *et al*., 2010; PANESAR, KUMARI & PANESAR, 2010; BAYRAMOGLU, TUNALI & ARICA, 2007).

A reação de transgalactosilação, e a consequente formação de GOS, foi observada no início dos anos 50 (WALLENFELS, 1951). Goslin *et al*. (2010); Torres *et al*. (2010) e Mahoney (1998) descreveram minuciosamente as características e cinética desta reação. Nesta revisão serão enfatizados alguns dos aspectos mais importantes abordados na literatura especializada, visando colaborar para o entendimento da produção de GOS através de diversos processos biotecnológicos.

A conversão da lactose em GOS por ação da enzima β-galactosidase é uma reação cineticamente controlada e responde a um modelo de competição entre a reação de transgalactosilação e hidrólise. O mecanismo bioquímico de síntese de GOS é complexo e

pode ser resumido da seguinte forma: o primeiro passo é a formação de um complexo enzima-galactosil e a simultânea liberação da glicose. Em uma segunda etapa o complexo enzima-galactosil é transferido para um aceptor que contenha um grupo hidroxil. Em uma solução com baixa concentração de lactose, água, ao invés de outros açúcares é mais competitiva para ser o aceptor deste complexo, sendo liberada, neste caso, a galactose. Por outro lado, se a concentração de lactose no sistema for alta, as moléculas de lactose e demais mono, di e oligossacarídeos, têm mais chances de atuarem como aceptores do complexo enzima-galactosil, formando, assim, os GOS (TORRES *et al*., 2010; MILICHOVÁ & ROSENBERG, 2006; ZHOU & CHEN, 2001).

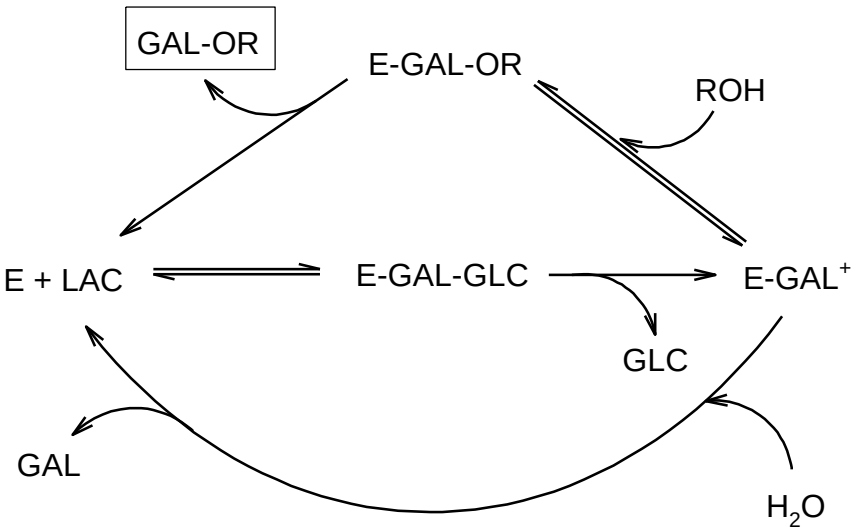

Figura 1: Mecanismo de reação proposto de β-galactosidase em lactose. E: enzima; LAC: lactose; GAL: galactose; GLC: glicose; ROH: aceptor de açúcar; GAL-OR: açúcar galactosil (galactooligossacarídeo). Fonte: Mahoney, 1998.

Não se elucidou, todavia, se a interação no sítio ativo da enzima é diferente de quando o aceptor é água ou é um sacarídeo. Contudo, sabe-se que β-galactosidases oriundas de fontes distintas, diferem quanto a seletividade à água e à moléculas de açúcar e que

mesmo em concentrações iguais de lactose inicial, resultam em rendimentos de GOS, estruturas e tipos de ligações glicosídicas diferentes (GOSLIN *et al*., 2010; OTIENO *et al*., 2010; BOON, JANSSEN & RIET, 2000). Para β-galactosidase tem sido estabelecido que as reações hidrolítica, sintética e de transferência são catalisadas pela mesma enzima (BECERRA *et al*., 2001; HUBER, KURZ & WALLENFELS, 1976; WALLENFELS & MALHOTRA, 1961).

Considerando o mecanismo de trasgalactosilação exposto acima, a relação entre a concentração inicial de lactose e a quantidade de GOS formada em um sistema, é constatada, na maioria dos estudos, como diretamente proporcional, pois esta contribui para elevar a quantidade de sacarídeos no meio e ao mesmo tempo diminui a disponibilidade de água, sendo aceita como um dos fatores mais influentes deste processo (GOSLIN et al., 2010). Contudo, é interessante ressalvar que alguns estudos empíricos de otimização da síntese de GOS observaram que a lactose não foi uma variável significativa na faixa de estudo analisada (CHEN, HSU & CHIANG, 2002; RUSTOM, FODA, & LÓPEZ-LEIVA, 1998). Goslin et al. (2010) sugerem que estes resultados podem apontar deficiências no modelo proposto e atualmente aceito para a reação de transgalactosilação.

A formação de GOS a partir da lactose é influenciada por outros fatores como a fonte e concentração de enzima, pH, temperatura, tipo de processo, entre outros (HELLEROVÁ & CURDA, 2009; ALMEIDA & PASTORE, 2004; BOON, JANSSEN & RIET, 2000). Os monossacarídeos glicose e, pricipalmente, galactose são reconhecidos como inibidores da reação de transgalactosilação. A inibiação da galactose parece ser resultado de uma competição com a lactose pelo sítio ativo da β-galactosidase, uma vez que esta enzima pode formar o complexo enzima-galactosil. O mecanismo de inibição pela

glicose é mais complexo e, dependendo da fonte da enzima, pode ser competitivo ou não competitivo (PARK & OH, 2010; GOSLIN *et al*., 2010).

Recentemente, estudos visando a otimização da produção de GOS através de metodologias experimentais de análise multivariada dos parâmetros reacionais, tais como análise de superfície de resposta, têm sido reportados (MANERA *et al*., 2010; MARTÍNEZ-VILLALUENGA *et al*., 2008; ALMEIDA & PASTORE, 2004; CHEN, HSU & CHIANG, 2002). Estes estudos vêm contribuindo para minimizar custos e tempo de reação, maximizar rendimento e produtividade, e contribuem para um maior entendimento da influência de cada variável no complexo mecanismo de síntese de GOS (DWEVEDI & KAYASTHA, 2009; RODRIGUES & IEMMA, 2005).

Quimicamente os GOS são formados por moléculas de galactose ligadas à glicose, sendo formados de tri a decassacarídeos com 2 a 9 unidades de galactose, respectivamente (LI *et al*., 2008).

Embora seja relatado que possam ser formados produtos transgalactosilados decassacarídeos, não é usual serem produzidos GOS com cadeias tão extensas (MUSSATTO & MANCILHA, 2007). De acordo com Mahoney (1998), a baixa efetividade da β-galactosidase em produzir GOS de maior peso molecular é explicada pela competitividade da reação de transgalactosilação com a de hidrólise. Entende-se assim, que o tempo de reação é um parâmetro crítico e tem direta influência na quantidade máxima e rendimento de GOS, uma vez que estes oligossacarídeos são simultâneamente sintetizados e degradados pela ação da β-galactosidase. Este autor afirma ainda, que a transgalactosilação será menos efetiva quanto maior o peso molecular do oligossacarideo aceptor, o que explicaria a maior formação de di, tri e tetrassacarideos em comparação com oligômeros maiores. A Tabela 1 apresenta as principais estruturas dos GOS.

Tabela 1: Principais estruturas químicas dos GOS formados a partir da lactose pela ação da enzima β-galactosidase. Gal: galactose; Glc: glicose. Fonte: Mahoney, 1998.

Dissacarídeos	β-D-Gal (1→6)-D-Glc	Alolactose
	β-D-Gal (1→6)-D-Gal	Galactobiose
	β-D-Gal (1→3)-D-Glc	
	β-D-Gal (1→2)-D-Glc	
	β-D-Gal (1→3)-D-Gal	
Trissacarídeos	β-D-Gal (1→6)-β-D-Gal (1→6)-D-Glc	6'dilactosil-glicose
	β-D-Gal (1→6)-β-D-Gal (1→4)-D-Glc	6'galactosil-lactose
	β-D-Gal (1→6)-β-D-Gal (1→6)-D-Gal	6'galactotriose
	β-D-Gal (1→3)-β-D-Gal (1→4)-D-Glc	3'galactosil-lactose
	β-D-Gal (1→4)-β-D-Gal (1→4)-D-Glc	4'galactosil-lactose
Tetrassacarídeos	β-D-Gal (1→6)-β-D-Gal (1→6)- β-D-Gal (1→4)-D-Glc	6'digalactosil-lactose
	β-D-Gal (1→6)-β-D-Gal (1→3)- β-D-Gal (1→4)-D-Glc	
	β-D-Gal (1→3)-β-D-Gal (1→6)- β-D-Gal (1→4)-D-Glc	
Pentassacarídeos	β-D-Gal (1→6)-β-D-Gal (1→6)- β-D-Gal (1→6)- β-D-Gal (1→4)-D-Glc	6'trigalactosil-lactose

Em termos de estrutura química, os GOS podem diferir em relação à seguintes características: composição, regioquímica, ligação glicosídica e grau de polimerização (Figura 2). Estas propriedades são dependentes do mecanismo do biocatalisador utilizado e das demais condições reacionais do processo (GOSLIN *et al*., 2010).

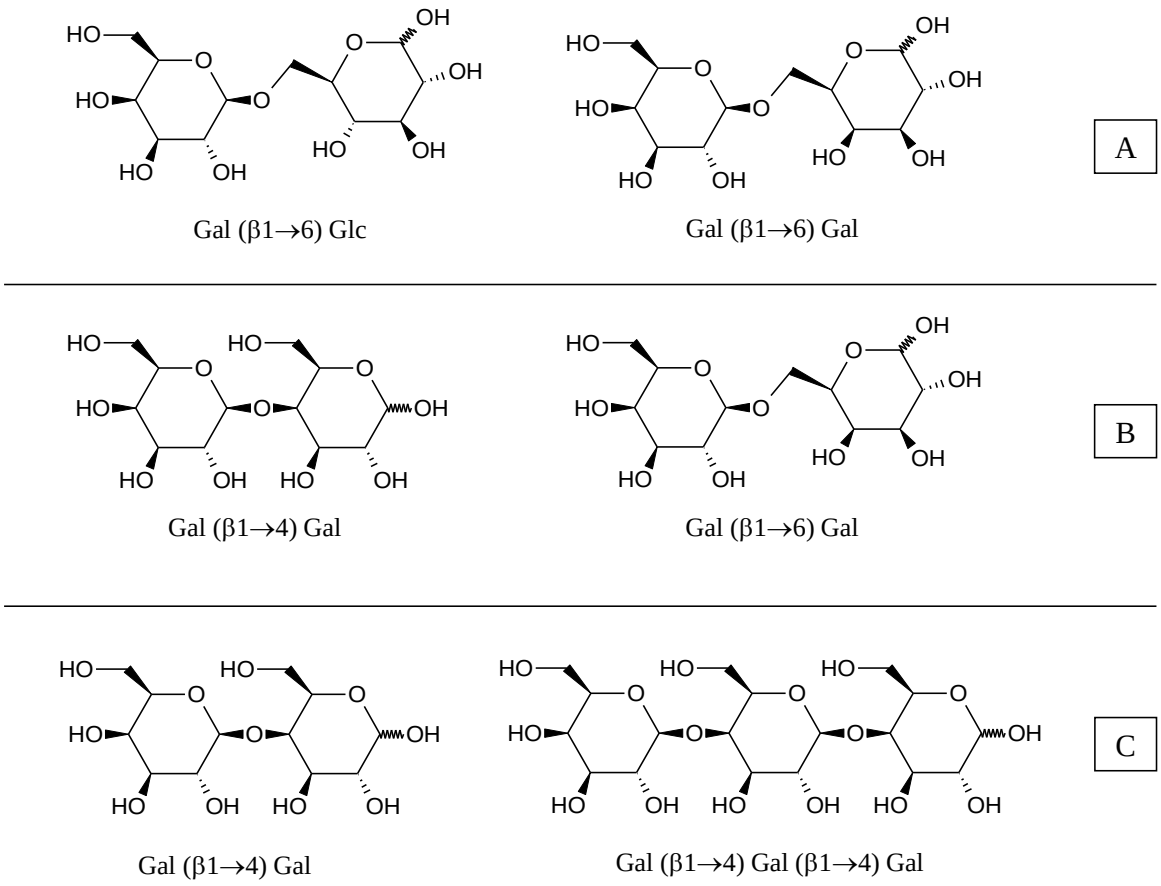

Figura 2: Exemplos de diferentes estruturas de GOS. (A) composição; (B) regioquímica; (C) grau de polimerização. Fonte: adaptado de Goslin *et al*. (2010).

É comum encontrar dissacarídeos transgalactosilados, consistindo em galactose e glicose com ligações β-glicosídicas diferentes da lactose ou com duas unidades de galactose (PARK & OH, 2010; SAKO, MATSUMOTO & TANAKA, 1999). É válido ressaltar que todos os tipos de GOS, incluindo os dissacarídeos transgalactosilados, são considerados oligossacarídeos não digeríveis e apresentam propriedades fisiológicas similares, apesar de algumas particularidades e especificidades em relação a alguns probióticos terem sido relatadas (CARDELLE-COBAS *et al*., 2011; ALBAYRAK & YANG, 2002).

Um ponto interessante é levantando por Rastall (2004) o qual sugere que a biotecnologia de produção de GOS seja aperfeiçoada sintetizando estes oligossacarídeos a partir de β-galactosidases dos próprios micro-organismos probióticos, partindo do princípio de que o prebiótico formado seria um substrato com uma especificidade e biodisponbilidade maior para este grupo microbiano.

Considerando que tanto a estrutura do oligossacarídeo, como a espécie probiótica, são fatores importantes e sinérgicos para o equilíbrio da microbiota intestinal, tem-se investigado a produção de oligossacarídeos alvo a partir de culturas probióticas. Como resultado, geram-se simbióticos de alta eficiência baseados no binômio micro-organismo-substrato ideal (DEPEINT *et al*., 2008; GOULAS, TZORTZIS & GIBSON, 2007; TZORTZIS *et al*., 2004; TZORTZIS *et al*., 2003; COLLINS & GIBSON, 1999).

2.1 β-galactosidase

A enzima β-galactosidase (β-D-galactosideo-galactohidrolase, E.C.3.2.1.23) é usualmente chamada de lactase uma vez que esta catalisa a hidrólise da lactose em seus açúcares constituintes (PARK & OH, 2010).

Todas as enzimas denominadas genericamente como lactases são β-galactosidases, mas o inverso não é verdadeiro. Determinadas β-galactosidases, incluindo algumas de células vegetais e de órgãos de mamíferos, que não as do intestino, têm baixa ou até mesmo nenhuma atividade de hidrólise da lactose uma vez que a função catalítica das mesmas é a quebra de outros grupos galactosil, tais como glicolipídios, glicoproteínas e mucopolissacarídeos (MAHONEY, 2003).

Conforme mencionado anteriormente, esta enzima é igualmente responsável pela síntese de GOS, quando há uma alta concentração de lactose no sistema, pois será favorecida a reação de transgalactosilação em detrimento à hidrólise, e quando este dissacarídeo se encontra em baixa concentração, ocorre sua hidrólise, resultando em glicose e galactose (NERI *et al*, 2009b).

β-galactosidase é uma das enzimas mais pesquisadas e relatadas na literatura e ocorre em plantas como amêndoas, damascos, pêssegos, pêras, entre outros, em órgãos de animais como intestino e ainda é produzida por uma vasta gama de micro-organismos (MAHONEY, 2003; DWEVEDI & KAYASTHA, 2009).

A fonte preferida desta enzima para aplicações biotecnológicas é a microbiana, incluindo fungos (SANTOS, SIMIQUELI & PASTORE, 2009; ZHENG *et al*., 2006), leveduras (PETROVA & KUJUMDZIEVA, 2010; ISHIKAWA *et al*., 2005) e bactérias (GOSLIN *et al*., 2009; SPLECHTNA, NGUYEN, HALTRICH, 2007) e as condições ótimas do processo, seja de hidrólise ou de síntese, variam de acordo com o micro-organismo em que esta foi extraída (MACFARLANE & MACFARLANE, 2008; MEDEIROS *et al*., 2008).

Esta enzima apresenta uma importância comercial relevante, uma vez que, além da propriedade de produzir GOS, ao catalisar a hidrólise da lactose, sana problemas associados com a eliminação do soro, cristalização deste dissacarídeo em alimentos congelados e o consumo de leite e derivados por indivíduos com intolerância à lactose (DWEVEDI & KAYASTHA, 2009; GROSOVÁ, ROSENBER & REBROS, 2008).

A intolerância à lactose e a má digestão deste dissacarídeo consiste na ausência ou deficiência na produção da lactase e este é um problema que atinge mais de 50% da população mundial (LOMER, PARKES & SANDERSON, 2008; MLICHOVÁ &

ROSENBERG, 2006; VESA, MARTEAU & KORPELA, 2000; ENWONWU & SCRIMSHAW, 1993). A produção da lactase reduz-se após a fase inicial da vida, porém, em alguns grupos populacionais, em particular nas regiões do norte europeu, ocorreram desvios genéticos, persistindo a atividade desta enzima, portanto, a tolerância à lactose se desenvolveu como uma mutação genética (CUNHA *et al*., 2007; SWALLOW, 2003). Apesar de não existirem dados oficiais, estima-se que mais de 58 milhões de brasileiros sejam intolerantes à lactose (MARTINS & BURKERT, 2009).

Deve-se observar que a lactose também pode ser hidrolisada por ácidos, contudo, a temperatura necessária é extremamente maior (150°C) que quando se utilizada β-galactosidase (30-50ºC). A temperatura excessivamente alta acarreta no escurecimento dos produtos e na formação de compostos de aroma indesejáveis, tornando-os inapropriados ao uso em alimentos. Por outro lado, a hidrólise enzimática mantém as características nutricionais e de qualidade desejadas dos produtos, além de aumentar a doçura dos mesmos. Percebe-se assim que a clivagem da lactose via enzimática é muito mais conveniente à indústria de alimentos (GROSOVÁ, ROSENBERG & REBROS, 2008).

Como consequência, percebe-se um crescimento considerável na pesquisa e desenvolvimento de processos biotecnológicos, economicamente viáveis, para a obtenção de produtos lácteos com baixo teor de lactose (RODRIGUEZ, CRAVERO & ALONSO, 2008; LADERO, SANTOS & GARCIA-OCHOA, 2000).

2.1.1. Imobilização enzimática

Existem basicamente duas formas de utilização de β-galactosidase, seja para hidrólise da lactose ou síntese de GOS: na forma solúvel (livre) e imobilizada, normalmente

operando em processos de batelada e contínuo, respectivamente (HAIDER & HUSAIN, 2009). Biocatalizadores imobilizados, enzimas ou células, são catalisadores fisicamente confinados ou localizados em uma região definida do espaço, com retenção de suas atividades catalíticas, e que podem ser utilizados repetida ou continuamente (CHIBATA, 1978; KATCHALSKI-KATZIR & KRAEMER, 2000; CARVALHO, CANILHA & SILVA, 2006).

A opção de imobilização é principalmente induzida pela facilidade de controle da reação e possibilidade de reutilização do sistema, além de aumentar a estabilidade do biocatalizador em questão e permitir o escalonamento industrial (CANILHA, CARVALHO & SILVA, 2006; NERI *et al* 2009a).

Verifica-se na literatura um grande número de publicações visando a produção de GOS e hidrólise da lactose, contemplando diferentes matrizes e técnicas, tais como: *cross-linking* (ZHANG *et al*., 2006), adsorção (GAUR *et al*., 2006; SHIN, PARK & YANG, 1998), ligação covalente (ELNASHAR & YASSIN 2009; NERI *et al*., 2009 b; BIRÓ *et al*., 2008; MARIOTTI *et al*., 2008), ligação iônica (PESSELA *et al*., 2003), encapsulamento (WU *et al*., 2010), ultrafiltração (EBRAHIMI *et al*., 2010), e, até mesmo, a combinação entre esses métodos (ANSARI & HUSAIN, 2010; PURI *et al*., 2010; HAIDER & HUSAIN, 2009).

Panesar, Kumari & Panesa (2010) revisaram de forma abrangente este assunto e ressaltam o atual interesse e importância em se encontrar um método adequado para a imobilização de β-galactosidase, extraída previamente ou em células microbianas.

Ressalta-se aqui que a escolha de um sistema enzimático adequado depende tanto das propriedades deste biocatalizador quanto da finalidade de uso do mesmo. Por exemplo, β-galactosidases extraídas de leveduras são geralmente utilizadas na hidrólise de leite e

soro doce, enquanto as de fungos são mais adequadas para aplicação em soro ácido. Comparada com β-galactosidases sintetizadas por leveduras as de fungo são mais termoestáveis, por outro lado, são mais sensíveis frente aos inibidores do produto final, principalmente a galactose (GROSOVÁ, ROSENBERG & REBROS, 2008).

Gaur *et al*. (2006) compararam três técnicas distintas de imobilização de β-galactosidase de *Aspergillus oryzae*: por adsorção em celite, por ligação covalente em quitosana e por formação de *agregados* com ligações cruzadas. Os autores concluíram que a formação de ligação covalente da enzima em quitosana foi mais apropriada para a síntese de oligossacarídeos, enquanto a adsorção e formação de agregados foram mais adequadas para a hidrólise de lactose.

Uma técnica para produção de não-monossacarídeos e galactooligossacarídeos de alta pureza, a partir de lactose, foi desenvolvida utilizando β-galactosidase de *Penicillium expansum* imobilizada em alginato de cálcio juntamente com células de *Saccharomyces cerevisiae* ou *Kluyveromyces lactis* neste suporte. Neste caso, o GOS sintetizado, a partir da enzima imobilizada, ficava disponível para fermentação pelas leveduras, as quais consumiam a glicose e galactose remanescente, resultando em aumento de pureza do GOS produzido em até 97,5% (LI *et al*., 2008).

Becerra *et al*. (2001) desenvolveram um sistema de biocatálise a partir de células de *Kluyveromyces lactis* imobilizada em alginato de cálcio e constataram que a atividade de β-galactosidase por unidade de biomassa celular foi maior com células imobilizadas do que com células livres, no mesmo meio de cultura. Estas células imobilizadas foram capazes de hidrolisar, sem produção simultânea de etanol, aproximadamente 99,5% de lactose à 30 ºC em 30 h.

2.3 Utilização de células para a produção de GOS

Assim como a maioria dos compostos provenientes de processos biotecnológicos, os GOS podem ser sintetizados de diversas maneiras, sendo a fermentação uma opção interessante. Neste caso, a síntese destes oligossacarídeos é levada a cabo utilizando diretamente micro-organismos (sem extração prévia da β-galactosidase) que produzam esta enzima, por fermentação de substratos com alta concentração de lactose (GOSLIN *et al*., 2010; KIM, LEE & LEE, 2001).

A fermentação apresenta como vantagens, em relação a utilização de enzimas isoladas em processos biotecnológicos, o fato de não necessitar das etapas de isolamento e purificação da enzima, as quais podem ser onerosas, fastidiosas e demandar muito tempo (GOSLIN *et al*., 2010; GOULAS, TZORTZIS & GIBSON, 2007).

Além do mais, o uso de células viáveis neste tipo de processo pode contribuir com interessantes funções metabólicas adicionais, como o consumo dos monossacarídeos glicose e galactose provenientes da reação de transgalactosilação e hidrólise. Estes monômeros não apresentam efeito prebiótico, aumentam o aporte calórico deste ingrediente e sua remoção resultam em GOS com mair teor de pureza (PARK & OH, 2010; GOULAS *et al*., 2007).

Em contrapartida, há a necessidade de se manter o processo em condições estéreis para que não haja contaminação no sistema e o produto de interesse seja efeitvamente acumulado (GOSLIN *et al*., 2010; LEE, KIM & OH, 2004).

Contudo, existem poucos estudos na literatura nos quais estes oligosscarídeos transgalactosilados tenham sido produzidos por fermentação. Onishi, Yamashiro & Yokozeki (1995) produziram GOS por fermentação da lactose a partir de culturas de *Bacillus circulans*, *Rhizobium meliloti*, *Rhodotorula minuta*, *Sirobasidium magnum*,

Sterigmatomyces elviae, entre outros, e obtiveram 57; 42; 67; 63 e 74 g/L de GOS total sintetizado, respectivamente, partindo de uma concentração inicial de lactose de 30% (p/v) à 30 °C/16 h.

Roy, Daoudi & Azaola (2002) otimizaram a síntese de GOS fermentado por *Bifidobacterium infantis*, a partir da lactose, utilizando metodologia de superfície de resposta e alcançaram um máximo de 43% (v/v) de rendimento de GOS em relação a concentração incial de lactose de 400 g/L.

Onishi & Tanaka (1998) desenvolveram um método de reciclo celular de *Sterigmatomyces elviae* no qual a cultura se manteve viável e estável por 6 ciclos, alcançando um alto rendimento de GOS, em torno de 60% (v/v), a partir de uma concentração inicial de lactose de 360 g/L.

Outra metodologia que merece ser comentada e que tem ganhado destaque atualmente é a de síntese de GOS e a hidrólise da lactose utilizando células permeabilizadas (MANERA *et al*., 2011; KAUR *et al*., 2008).

Em alguns micro-organismos a β-galactosidase é uma enzima intracelular, o que resulta em algumas limitações na sua aplicação. Para a obtenção de extratos livres destas céulas faz-se necessário a ruptura das mesmas, o que pode levar à inativação da enzima. A permeabilização celular é apontada como um método alternativo neste caso; esta técnica modifica a estrutura da membrana do micro-organismo, diminuindo o conteúdo de fosfolipídios e facilitando assim, a entrada e saída de solutos, tais como a lactose e seus produtos reacionais, ou seja, aumenta a permeabilidade da célula. Outro aspecto interessante é que as enzimas intracelulares, das células permeabilizadas, podem ser consideradas "naturalmente" imobilizadas (MANERA *et al*., 2010; PARK & OH, 2010; PANESAR, 2008).

Kaur *et al*. (2008) estudaram as variáveis que afetam o processo de hidrólise da lactose com células permeabilizadas de *Kluyveromyces marxianus*, utilizando brometo de cetiltrimetil amônio como agente permeabilizante, e alcançaram um máximo de 90,5% de hidrólise após 90 minutos à 40°C.

Manera *et al*. (2010) utilizaram uma estratégia sequencial de planejamentos para produção de GOS a partir de células de *Kluyveromyces marxianus* permeabilizadas com isopropanol. Nas condições otimizadas, com uma concentração de lactose de 50% (p/v), os autores constataram rendimento e produtividade de 16,5% e 27,6 g/L.h, respectivamente.

Onishi, Kira & Yokozeki (1996) investigaram a produção de GOS empregando células de *Sirobasidium magnum*, permeabilizadas com tolueno, obtendo rendimento de 37% a partir de uma solução de lactose de 360 g/L em 42 h à 50°C.

3. Efeitos benéficos à saúde

A característica prebiótica dos GOS é amplamente estudada e corroborada na literatura sob diversas condições experimentais *in vitro* e *in vivo* (OOI & LIONG, 2010; VEEREMAN-WAUTERS, 2005; TZORTZIS *et al*., 2005). A terminação "prebióticos" foi introduzida por Gibson & Roberfroid (1995) que definiram como prebiótico aquele componente não digerível dos alimentos e que resulta em benefício para o hospedeiro, uma vez que estimula as bactérias benéficas do cólon intestinal.

Neste sentido, os critérios para se definir um prebiótico são: resistência à digestão no intestino delgado, hidrólise e fermentação pelas bactérias desejáveis no cólon e estimulação seletiva do crescimento desta mesma microflora, ou efeito bifidogênico (PÉRIS & GIMENO, 2008; BARRETEAU, DELATTRE & MICHAUD, 2006).

O mecanismo de atuação fisiológica dos prebióticos ocorre da seguinte maneira: assim como as demais fibras da dieta, são resistentes à digestão na parte superior do trato intestinal, sendo subsequentemente fermentados no cólon (TUNGLAND & MEYER, 2002). Exercem, então, um efeito de aumento de volume, como consequência do aumento da biomassa microbiana resultante de sua fermentação, promovendo, ainda, um aumento na frequência de evacuações, efeitos estes que confirmam a sua classificação no conceito atual de fibras da dieta (TUOHY *et al*., 2003; KAUR & GUPTA, 2002; ROBERFROID, 2002).

Os benefícios da ingestão de galactooligossacarídeos são o aumento da população de bifidobactérias no cólon e por efeito antagônico, supressão da atividade de bactérias putrefativas reduzindo a formação de metabólitos tóxicos (TOMOMATSU, 1994; NERI *et al*, 2009a).

A mudança da microflora intestinal é apontada como responsável pela diminuição de produtos putrefativos nas fezes, por diminuir o conteúdo de colesterol no sangue (PERIS & GIMENO, 2002) e reduzir a incidência de câncer colorretal (RIVERO-URGELL & SANTAMARIA-ORLEANS, 2001; WIJNANDS *et al*., 2001).

Também demonstram benefícios derivados da sua atividade antiadesiva, visto que inibem infecções por patógenos entéricos impedindo a adesão destes micro-organismos às células epiteliais gastrointestinais (GIESE *et al*., 2011; SEARLE et al., 2010). É relatado, ainda, efeito protetor contra infecções no trato urogenital (SOUSA, SANTOS & SGARBIERI, 2011; MUSSATTO & MANSILHA, 2007).

Vários relatos sugerem também que os GOS, dentre outros carboidratos não digeríveis, estimulam e aumentam a absorção de cálcio e minerais no intestino. Este efeito consiste na produção de ácidos graxos de cadeia curta, resultante da fermentação no

intestino grosso, uma vez que eles estimulam a proliferação de células do epitélio do intestino e reduzem o pH luminal (SANTOS *et al*., 2011; SCHOLZ-AHRENS, 2007).

4. Propriedades e aplicação na indústria

Os GOS disponíveis comercialmente, na forma líquida ou em pó, são compostos por vários tipos de misturas de oligossacarídeos transgalactosilados mesclados à lactose, glicose e galactose resultantes do processo de síntese e hidrólise (LI *et al*., 2008).

De forma geral, os oligossacarídeos são solúveis em água, levemente doces e equivalem de 30 a 60% a doçura da sacarose. O grau de doçura é dependente da estrutura química e da massa molar dos oligossacarídeos, bem como da concentração de mono e dissacarídeos presentes na mistura. Quanto maior a peso molecular, menor a doçura (PLAYNE & CRITTENDEN, 2009; MUSSATTO & MANCILHA, 2007).

Comparado aos mono e dissacarídeos, o maior peso molecular dos oligossacarídeos promove ainda um incremento da viscosidade, almejado em diversos produtos. Também podem ser usados para alterar a temperatura de alimentos congelados e controlar o nível de escurecimento devido a reação de Maillard em alimentos processados com o uso de altas temperaturas. Promovem também uma retenção da umidade prevenindo a excessiva secagem de alguns alimentos, e diminuem a proporção de atividade de água livre, sendo conveniente no controle de contaminação microbiana (MUSSATTO & MANCILHA, 2007; PLAYNE & CRITTENDEN, 2002; CRITTENDEN & PLAYNE, 1996).

Os GOS podem ser usados largamente na produção de doces, massas, pães e geléias, haja vista que são termo e quimicamente estáveis em uma ampla faixa de pH (MLICHOVÁ & ROSENBERG, 2006; ALMEIDA & PASTORE, 2004). Resistem a 160

°C por 10 min em pH neutro ou a 120 °C por igual tempo em pH 3. Em pH 2 chegam a resistir até a 100 °C por 10 min. Nesta condição adversa a degradação da sacarose ulrapassaria 50% (MARTINS & BURKERT, 2009; SAKO, MATSUMOTO & TANAKA, 1999).

Uma vez que os GOS estão presente no leite humano algumas empresas têm utilizado estes oligossacarídeos como aditivos em formulações infantis no intuito de mimetizar o efeito bifidogênico comprovado no leite materno aos demais produtos para esta faixa etária (SCHWAB & GANZLE, 2011; RIVERO-URGELL & SANTAMARIA-ORLEANS, 2001; MLICHOVÁ & ROSENBERG, 2006).

Ao contrário do amido e dos monossacarídeos, os oligossacarídeos não são fermentados pela microflora bucal para formar ácidos e poliglucanas, sendo então utilizados como açúcares de baixa cariogenicidade em confeitos, gomas de mascar, iogurtes e bebidas (MUSSATTO & MANCILHA, 2007).

Outro ponto interessante é que, como não são digeríveis pelas enzimas digestivas, são açúcares de baixo valor calórico e podem ser aplicados em alimentos para diabéticos, podendo ainda ser utilizados em conjunto com edulcorantes artificiais, como aspartame e fenilalanina, mascarando o sabor indesejável produzido por alguns destes adoçantes (PLAYNE & CRITTENDEN, 2009; TOMOMATSU, 1994).

Dentre muitos produtos nos quais é possível a adição de GOS, como ingrediente bioativo, o pão é um alimento extremamente apropriado para a inclusão destes, pois durante a fermentação e o cozimento os GOS não são degradados e ainda favorecem o sabor e textura do mesmo. Produtos lácteos fermentados são outros bons exemplos e leites fermentados adicionados de GOS já estão comercialmente disponíveis. Alimentos infantis e especiais para idosos e imuno-deficientes são extremamente adequeados para a aplicação

de GOS, pois estes indivíduos são mais suscetíveis a alterações no equilíbrio intestinal, além de se beneficiarem das propriedades fisiológicas deste prebiótico em condições especiais (SAKO, MATSUMOTO & TANAKA, 1999).

Além da indústria de alimentos, outros setores como a indústria famacêutica e de cosméticos podem explorar as propriedades físico-químicas e fisiológicas dos GOS e atualmente já se observam avanços neste sentido (TORRES *et al*., 2010).

É interessante destacar que além dos benefícios fisiológicos que os GOS oferecem, estes são produtos de valor agregado uma vez que são produzidos a partir da lactose, substância extraída notadamente do soro de leite descartado, evitando assim, que este funcione como agente de poluição ambiental, devido à sua alta demanda biológica de oxigênio (BICAS *et al*., 2010; OTIENO *et al*., 2010; PANESAR *et al*., 2007).

Percebe-se assim, que os GOS são prebióticos comercialmente importantes, e hoje são manufaturados principalmente na Ásia e Europa, e mais recentemente na Austrália. No ano de 2005 e 2009 foi estimada a produção mundial máxima de quatorze mil e vinte um mil toneladas deste prebiótico, respectivamente (GOSLIN *et al*., 2010; PLAYNE & CRITTENDEN, 2009).

As principais empresas que manufaturam e comercializam GOS são: Yakult Honsha, Nissin Sugar Manufacturing, Snow Brand Milk Products no Japão, Friesland Foods Domo (antiga Borculo Domo ingredients), localizada na Holanda e Clasado Ltd situada no Reino Unido. Nos Estados Unidos a produção destes oligossacarídeos ainda é reduzida, apesar de existirem empresas neste setor, como a Corn Products International (TZORTZIS & VULEVIC, 2009). É interessante observar que algumas indústrias produzem GOS apenas com o intuito de incorporá-los em seus próprios produtos, sem comercializá-los diretamente como ingredientes (PLAYNE & CRITTENDEN, 2009).

Geralmente, os GOS são comercializados como xaropes transparentes ou em pó e, independente da forma, se apresentam como misturas de GOS com diversos graus de polimerização junto à lactose, glicose e galactose residuais. Os GOS comercializados entre indústrias diferentes, e até mesmo dentro de uma mesma unidade, se diferenciam quanto ao grau de pureza, peso molecular, tipos de ligações glicosídicas e em alguns casos, finalidade de aplicação. Estas diferenças são inerentes aos variados processos de obtenção destes oligossacarídeos (TORRES *et al*., 2010; PLAYNE & CRITTENDEN, 2009).

A Yakult produz três tipos de GOS: Oligomate 55 na forma de xarope, Oligomate 55P em pó e TOS-100 uma versão de alta pureza, contendo 99% de oligossacarídeos. A Nissin produz os GOS com as marcas comerciais Cup-Oligo H70 e Cup-Oligo P na forma de xarope e em pó, respectivamente, enquanto a Snow Brand fabrica GOS apenas com a finalidade de adicioná-lo no leite infantil que estes produzem, sem a intenção de comercializá-lo diretamente. Na Europa, a Friesland Food Domo comercializa este prebiótico com a marca de Vivinal GOS, o qual contém aproximadamente 57 e 29% (w/w) de oligossacarídeos na forma de xarope e em pó, respectivamente. A Clasado Ltd, por sua vez, comercializa o produto Bimuno, GOS contendo 52% (w/w) de oligossacarídeos nas versões de xarope e em pó. Com exceção do GOS TOS-100 altamente purificado fabricado pela Yakult, os GOS comercializados atualmente apresentam de 40 a 70% de oligossacarídeos em sua composição (TZORTZIS & VULEVIC, 2009).

É patente que informações detalhadas sobre o processo de fabricação destes oligossacarídeos são escassas. Sabe-se que o produto Oligomate é sintetizado por β-galactosidase de *Aspergillus oryza*e e apresenta, principalmente, ligações do tipo β 1-6 e que a Yakult está realizando estudos para utilizar uma combinação das enzimas extraídas de *A. oryzae* and *Bacillus circulans*, com o intuito de fabricar oligossacarídeos com

ligações β 1-4 e 1-6. Os produtos Cup-Oligo e Vivinal apresentam, especialmente, ligações do tipo β 1-4, como resultado da síntese por β-galactosidase de *Cryptococcus laurentii* e *B. circulans*, respectivamente, enquanto o GOS da marca Bimuno é produzido pela enzima de *Bifidobacterium bifidum* e apresenta preferencialmente ligações do tipo β 1-3 (TZORTZIS & VULEVIC, 2009).

Ainda que utilizem enzimas de fontes microbianas distintas, o que é relatado acerca do fluxograma de processamento de GOS por estas indústrias, é semelhante. A concentração inicial de lactose varia entre 20 a 40% (w/v) a qual é incubada com a enzima em reator de batelada ou contínuo até o ponto ótimo de conversão de lactose em GOS ser alcançado. A solução é então descolorida, desmineralizada e submetida a uma etapa de separação dos monossacarídeos para purificação do produto. A solução resultante é concentrada usualmente até 67-75% de sólidos totais. Parte destes processos e outros para a produção de GOS estão protegidos por patentes de invenção (PLAYNE & CRITTENDEN, 2009).

Apesar de apresentarem interessantes características físico-químicas, não há dúvidas de que o principal interesse do uso dos oligossacarídeos prebióticos como ingredientes nos alimentos se deve às suas propriedades fisiológicas. Assim, à medida que os diversos efeitos benéficos dos GOS são cientificamente esclarecidos e deslocados para diante, uma maior demanda por estes produtos é evidenciada (ROBERFROID, 2007; TUOHY *et al*., 2003; NAKAKUKI, 2002).

5. Perspectivas e avanços futuros

As recentes comprovações científicas estreitando a relação entre dieta e saúde, aliada à maior expectativa de vida e a valorização do consumidor por alimentos saudáveis trouxeram um novo desafio para a indústria de alimentos: o de se adaptar à era da nutrição otimizada.

Por outro lado, os alimentos funcionais configuram-se não somente como a nova fronteira da ciência e tecnologia de alimentos, mas como atributos positivos para criar novos mercados (RAUD, 2008).

Consoantes aos aspectos acima mencionados há uma ampla variedade de aplicações para os GOS como ingredientes a serem exploradas na indústria de alimentos, permitindo agregar benefícios nutricionais e sensoriais a alimentos já existentes e novos produtos.

Considerando que os GOS são sintetizados a partir de reações enzimáticas, seja pela extração prévia do biocatalisador ou utilizando diretamente a fonte microbiana da enzima para fermentação da lactose, avaliasse que o advento da inovação biotecnológica na área de enzimas e fermentações impacte diretamente sobre a pesquisa e produção comercial destes açúcares não convencionais, dentre outros produtos deste gênero.

Espera-se um expressivo avanço na área de produção de enzimas em âmbito nacional como consequência da promulgação da Política de Desenvolvimento da Biotecnologia no Brasil através do decreto 6.041/07 (BRASIL, 2007). Este marco regulatório, quando trata da biotecnologia industrial, ressalta de forma explícita a tecnologia enzimática como uma de suas áreas prioritárias. A implantação da tecnologia enzimática no Brasil beneficiará setores industriais das áreas de alimentos, química, farmacêutica, de biocombustíveis, entre outros, já consolidados ou em processo de

estabelecimento no país, e carentes de inovação para valorização e diversificação de seus produtos e processos (BON *et al*., 2008).

Especificamente no Brasil o avanço da tecnologia enzimática é ainda favorecido por existir conhecimento das tecnologias para produção de enzimas em larga escala, de processos fermentativos e extrativos, bem como a maior biodiversidade do planeta como fonte de biocatalizadores. De forma complementar, há uma notável biodiversidade microbiana a ser estudada, para aplicação imediata ou após melhoramento genético, visando a produção de compostos de crescente interesse, tais como os oligosscarídeos, por processos fermentativos e enzimáticos (BON *et al*., 2008).

Estima-se que o setor de alimentos funcionais movimente cerca de 50 bilhões de dólares no mundo e apresente um ritmo de crescimento de cerca de 10% ao ano, índice três vezes maior que o de produtos alimentícios convencionais. A previsão é de que em dez anos os funcionais detenham 40% do mercado de alimentos (RAUD, 2008; COSTA, 2007).

A projeção do segmento para o ano de 2015, elaborada pela *Global Industry Analysts*, para ingredientes prebióticos na Europa e nos Estados Unidos é de aproximadamente 1,2 bilhões e 225 milhões de dólares, respectivamente (NUTRACEUTICALS WORLD, 2010).

Alimentos, saúde e biotecnologia já se apresentam no presente e assim persistirão, como um trinômio indissociável e essencial para que a indústria atenda as exigências de uma sociedade que busca por uma alimentação mais saudável e novos modos de vida.

6. Considerações finais

As forças condutoras que promovem a constante pesquisa por processos eficientes para a produção de ingredientes bioativos, incluem as exigências de uma sociedade que entende o papel primordial da alimentação na manutenção da saúde e prevenção de doenças. Os efeitos fisiológicos dos GOS, e demais oligossacarídeos, certamente continuarão a ser elucidados em ensaios *in vitro* e *in vivo* nos próximos anos. A combinação desta acepção, aliada ao crescente número de estudos visando a otimização da síntese de GOS, estimula também o setor industrial a aplicar recursos para a produção destes compostos. Assim, estima-se que haja um aumento expressivo da aplicação destes ingredientes bioativos, em alimentos de consumo usual e em produtos específicos, em um futuro próximo.

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Capítulo 2

SYNTHESIS OF GALACTOOLIGOSACCHARIDES FROM LACTOSE BY β-GALACTOSIDASE IMMOBILIZED ON GLUTARALDEHYDE-TREATED CHITOSAN

Resumo

Síntese de galactooligossacarídeos, a partir da lactose, por β-galactosidase imobilizada em quitosana ativada com glutaraldeído.

β-galactosidase de *Aspergillus oryzae* foi covalentemente imobilizada em quitosana em pó, ativada com glutaraldeído, e suas propriedades foram avaliadas e comparadas àquelas da enzima livre. Uma coluna encamisada empacotada com a enzima imobilizada, com reciclo de lactose, foi utilizada para a produção de galactooligossacarídeos e hidrólise da lactose. O pH ótimo para a enzima na forma livre e imobilizada foi de 4,6 e 5,0, respectivamente. A temperatura ótima constatada para a enzima livre foi de 40 °C, sendo 10 °C acima para a enzima imobilizada. A enzima β-galactosidase imobilizada apresentou boa estabilidade operacional quando utlizada em dez repetições. A produtividade máxima de galactooligossacarídeos foi de 14,42 g/L.h a partir de 400 g/L (p/v) de uma solução de lactose, após duas horas de reação. A lactose foi 44% hidrolisada em 12 horas.

Palavras-chaves: β-galactosidase, prebiótico, reação de transgalactosilação, quitosana, ligação covalente.

SYNTHESIS OF GALACTOOLIGOSACCHARIDES FROM LACTOSE BY β-GALACTOSIDASE IMMOBILIZED ON GLUTARALDEHYDE-TREATED CHITOSAN

ABSTRACT

Aspergillus oryzae β-galactosidase was covalently immobilized on glutaraldehyde-treated chitosan powder and its properties were evaluated and compared with those of the free enzyme. A fixed-bed reactor with lactose recycle was employed for production of galactooligosaccharides synthesis and lactose hydrolysis by immobilized enzyme. The optimum pH for soluble and immobilized β-galactosidase were 4.6 and 5.0, respectively. The optimum temperature for the free enzyme was 40°C, yet this value was 10°C higher when characterizing the immobilized enzyme. Immobilized β-galactosidase had a good operational stability when used 10 times repeatedly and galactooligosaccharides synthesis had a maximum productivity of 14.42g/L.h from 400 g/L (w/v) lactose solution after two hours of reaction. Lactose was 44 % hydrolyzed in 12 hours.

Keywords: β-galactosidase; prebiotic; transgalactosylation reaction; chitosan; covalent binding; semicontinuous process.

1. INTRODUCTION

Galactooligosaccharides (GOS), containing 3–10 molecules of galactose and glucose, selectively stimulating the beneficial colonic microflora, impart physiological

benefits to the consumer and are among the most promising prebiotics (LI, LU & LI, 2008; GOSLIN *et al.*, 2009).

GOS can be produced from lactose by enzymatic transgalactosylation using β-galactosidase (E.C.3.2.1.23) of great technological interest from various yeast (MANERA *et al*., 2011; PETROVA & KUJUMDZIEVA, 2010), fungi (HUERTA *et al*., 2011; LI, LU & LI, 2008; SANTOS, SIMIQUELI & PASTORE, 2009; ZHENG *et al*, 2006) and bacteria (GOSLING et al., 2009; SPLECHTNA *et al*., 2007; CHEN, YANG & YEH, 2003).

The biochemical mechanism of β-galactosidase is highly relevant to the synthesis of GOS products and has been thoroughly described (GOSLIN *et al*., 2010). A brief summary of the critical catalytic events of the enzyme is described as follows: the first step is the enzyme–galactosyl complex formation and simultaneous glucose liberation. In the second step, the enzyme–galactosyl complex is transferred to an acceptor containing a hydroxyl group. In a diluted lactose solution water, rather than other sugars, become more competitive to be an acceptor, therefore galactose is formed and released from the active site. On the other hand, in a high lactose content solution, lactose molecule has more chances to act as the acceptor, binding with the enzyme–galactose complex (ZHOU & CHEN, 2001a).

Thus, converting lactose into GOS by *β*-galactosidases is a kinetically controlled reaction, by means of the competition between hydrolysis and transgalactosylation. Specifically, during this conversion, the thermodynamically favored hydrolysis of lactose, which generates galactose and glucose, competes with the transferase activity that generates a complex mixture of several GOS of different structures (TORRES *et al*., 2010).

Biocatalytic process economics can be enhanced by enzyme reuse and the improvement in enzyme stability afforded by immobilization. The capacity to retain or

recover enzymes also allows biocatalyst separation from product (BRADY & JORDAAN, 2009). Nowadays, immobilized β-galactosidase is intensively being used in lactose hydrolysis of milk/whey and has been tested for the production of galactooligosaccharides (PANESAR, KUMARI & PANESA, 2010).

Of the many carriers that have been studied for enzyme and cell immobilization considerable attention has been paid to chitosan, a cationic, biodegradable, biocompatible, and bioactive amino polysaccharide, essentially composed of β-1,4 D-glucosamine (GlcNAc) linked to N-acetyl-D-glucosamine residues (ARANAZ *et al*., 2009).

Chitosan has been widely used as a support for enzyme immobilization due to its appropriate characteristics: high affinity to proteins, availability of reactive functional groups, mechanical stability and ease of preparation in different geometrical configurations (KRAJEWSKA, 2004).

In the present study, *Aspergillus oryzae* β-galactosidase was covalently attached, via glutaraldehyde, to powder chitosan to produce GOS and hydrolyze lactose. Bioconversion process kinetics and productivity were evaluated and compared with previously obtained data for β-galactosidase immobilized in various supports.

2. MATERIALS AND METHODS

2.1 Material

Aspergillus oryzae β-galactosidase (EC 3.2.1.23) and o-nitrophenyl-β-D-galactopyranoside (ONPG) was obtained from Sigma Co., USA. Comercial chitosan was purchased from Polymar, Brazil, with a deacetylation degree and apparent density of 91.1%

and 0.64 g/mL, respectively, according to the manufacturer's protocol. All the other reagents used were of analytical grade.

2.2 Preparation of immobilized enzyme

Chitosan in powder form was activated with glutaraldehyde 2.5% (v/v) in buffer acetate 0.2 M, pH 7.4 at 20 °C, 120 rpm, 30 min. The support was exhaustively washed with deionized water. β-galactosidase (5 mg) was then mixed with glutaraldehyde-activated chitosan (1.0 g suspended in 8 mL of 0.2 M acetate buffer, pH 5.0) and incubated overnight at 20 ºC under constant shaking. The preparation was repeatedly washed with buffer until no protein and enzyme activity were detected, when analyzed as described in sections 2.3 and 2.4. The chitosan-bound enzyme was suspended in 5 mL of the same buffer and used as an immobilized preparation for further studies. This methodology was adapted by Oliveira & Vieira (2006).

2.3 Protein estimation

Protein was determined by Bradford method (1976) using bovine serum albumin (BSA) as protein standard (GAUR *et al*., 2006).

2.4 β -Galactosidase assay

Activity of free and immobilized β-galactosidase was estimated by Food Chemical Codex method (FOOD CHEMICAL CODEX, 1981), using ONPG as substrate. Enzyme activity was defined as the amount of enzyme that liberated 1 µmol of o-nitrophenol (ONP) per minute under the standard assay conditions. An extinction coefficient for ONP of 4.3mM was calculated and used.

2.5 Effect of pH and temperature on enzyme activity

Optimum temperature and pH were determined by changing individually the conditions of the β-galactosidase activity assay: pH from 4 to 9 and temperature from 30 to 70ºC. Acetate buffer solution (0.2 M) was used for solutions at pH values between 4 and 6. Phosphate buffers (0.2 M) were used for values above this range (TANRISEVEN & DOGAN, 2002).

2.6 Determination of kinetic parameters Km and Vmax

The free and immobilized β-galactosidase were used to measure the kinetic using ONPG as substrate. Km and Vmax was determined using the Lineweaver–Burk plot method (ZHOU & CHEN, 2001b).

2.7 Batch production of galactooligosaccharides

Enzyme preparations were incubated with 40% lactose (w/v) solution in 0.2 M acetate buffer, pH 5.0 for 12 hours with intermittent stirring. The reaction was stopped by heating at 100 °C for 10 min. Oligosaccharides formed were analyzed by described in 2.9.

2.8 Semi-continuous synthesis of galactooligosaccharides

Fixed-bed reactor studies were carried out in a jacketed glass column with lactose recycle in order to produce galactooligosaccharides. The immobilized β-galactosidase (6.6 U/mg of support), obtained as previously described, was transferred to a jacketed column (2×8 cm) and to 40% lactose solution in 0.2 M acetate buffer (w/v), pH 4. This lactose solution circulated through a fixed-bed reactor in a recycle process using a MasterFlex® L/S peristaltic pump. Temperature of the column was maintained at 45 °C using an ultrathermostate Quimis® Q-14 M2 bath (precision temperature ±0.1°C). Sample aliquots

(1 mL) were collected at various times from 2 to 24 hours and kept in boiling water bath for 10 min. Oligosaccharides formed were analyzed by described in 2.9.

2.9 Estimation of oligosaccharides

The identification and quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD). A DIONEX (USA) chromatograph, supplied with a Carbopac PA1 (4x250 mm) column, a PA1 (4x50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and PEAKNET software were used for the analyses. Sugars were eluted with 20 mM sodium hydroxide, at a flow rate of 1.0 mL/min at room temperature. Before injection, the samples were diluted with water and filtered through 0.22 μm filters (MANERA *et al*., 2010).

3. RESULTS AND DISCUSSION

In this study *A. oryzae* β-galactosidase was covalently coupled to glutaraldehyde-treated chitosan and retained 55% of its original activity.

For kinetic parameters, pH, thermal, storage, and operational stability, both free and immobilized enzyme, were determined using ONPG. It is assumed that both reactions (synthesis and hydrolyze) are usually the same for these enzymatic parameters (BECERRA *et al*., 2001; HUBER, KURZ & WALLENFELS, 1976; WALLENFELS & MALHOTRA, 1961).

It was observed (Table 1) that optimum temperature for immobilized β-galactosidase was 10 °C higher when compared to the free enzyme which demonstrates that immobilized enzyme has increased temperature tolerance.

Table 1: Characterization of free and immobilized *Aspergillus oryzae* β-galactosidase.

Parameters	Free enzyme	Immobilized enzyme
Enzymatic activity (U/mg)	12.03 ± 0.22	6.6 ± 0.57
pH optima	4.6 ± 0.00	5.0 ± 0.00
Temperature optima (°C)	40 ± 0.00	50 ± 0.00
Km (mM)	2.34 ± 0.21	3 ± 0.60
Vmax (µmol ONP/min.mg)	43.47 ± 1.45	34.48 ± 2.14

In the literature, most immobilized β-galactosidase exhibited higher optimum temperature values than their free counterpart (HAIDER & HUSAIN, 2009; BAYRAMOGLU, TUNALI & ARICA, 2007; ZHOU & CHEN, 2001a). The increase in temperature tolerance may be due to diffusional effects and it may be explained by stating that the immobilization procedure could protect the enzyme active conformation from distortion or damage by heat exchange (GURDAS, GULEC & MUTLU, 2010).

Optimal pH was slightly affected by immobilization procedure and was shifted up 0.4 unit to a more alkaline value compared to its soluble form. The Michaelis–Menten constant Km was found to be increased approximately 1.3-folds after immobilization. When a biocatalyst is immobilized, kinetic parameters Km and Vmax undergo variations with respect to the corresponding parameters of the free form, revealing an affinity change for the substrate. These variations are attributed to several factors such as protein conformational changes induced by the support, steric hindrances and diffusional effects. These factors may operate simultaneously or separately, alternating the microenvironment around the bound enzyme (GURDAS, GULEC & MUTLU, 2010). The ratio Vmax/Km defines a measure of the catalytic efficiency of an enzyme-substrate pair (BAYRAMOGLU *et al*., 2011). In this study, catalytic efficiency of β-galactosidase was decreased about 1.62-fold upon immobilization.

This study was based on a simple and economical method to produce galactooligosaccharides and to hydrolyze lactose. Chitosan was used without changing its physical form, using the powder form to interact with glutaraldehyde and immobilize the enzyme. The enzyme load per gram of support was also economical (5 mg of β-galactosidase per gram of activated chitosan) when compared to other works (MARIOTTI *et al*., 2008; GAUR *et al*., 2006).

One of the limitations associated with enzyme industrial application is their high cost and instability under operational conditions. The overall process becomes cost-effective if the preparation shows higher efficiency, operational stability and reusability (GAUR *et al*., 2006). Chitosan covalent binding enzyme, in the present research, was reused for 10 cycles without significant loss in the enzymatic activity (retained at least 85% of its activity) and after 40 days of storage, suspended in 0.1 M acetate buffer, pH 4.5 at 3.5°C, retained 80% of its residual enzymatic activity.

It should be mentioned that it was not detected any microbial contamination by visual observation in the operational period of the packed bed reactor. However, when the immobilized enzyme was stored at 3 °C in acetate buffer after 40 days it was observed fungi contamination. Another observation is that the color of the chitosan powder changed from beige to brown after glutaraldehyde and enzyme preparation addition and there was no further change in color upon prolonged incubation. Similar results were found by Albayrak & Yang (2002) using cotton cloth as a support for β-galactosidase immobilization and it is suggested that the strength of the color seemed to be directly associated with the concentrations of glutaraldehyde and enzyme in the support. According to this authors the higher the concentrations of glutaraldehyde and enzyme, the darker the color was.

As seen from Figure 1, a higher rate of GOS formation was obtained at 50 °C compared with others temperatures, which was consistent with our expectation since this was the optima temperature observed in enzymatic activity assays performed with ONPG. However, the packed bed reactor was used at 45 °C to increase thermal stability for a longer period of time.

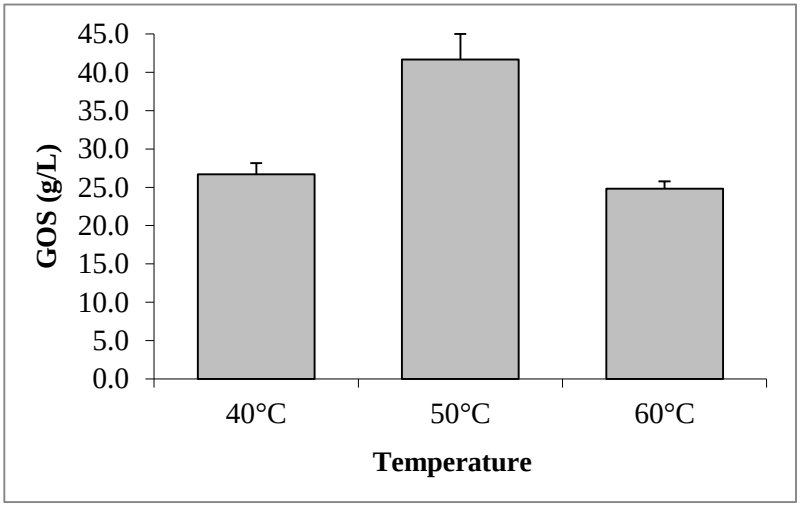


Figure 1: Effect of temperature on GOS production by immobilized enzyme in batch process from 40% (w/w) lactose, pH 5.0, 12 hours.

Time course of GOS production and lactose hydrolysis in fixed-bed reactor with lactose recycle were monitored and are shown in Figure 2 and 3, respectively.

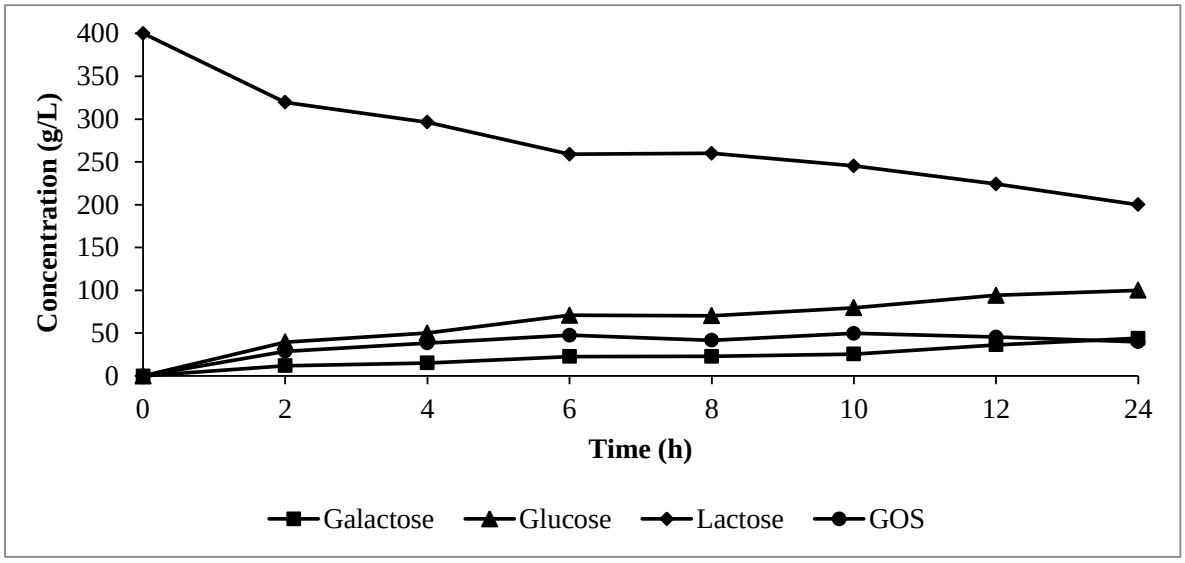


Figure 2: Reaction kinetics of GOS synthesis and lactose hydrolysis catalyzed by immobilized enzyme in the fixed-bed reactor in the first cycle, from 40% (w/w) lactose, pH 5.0, 45ºC. Coefficient of variation (CV) % = range of 0.28 to 5.09.

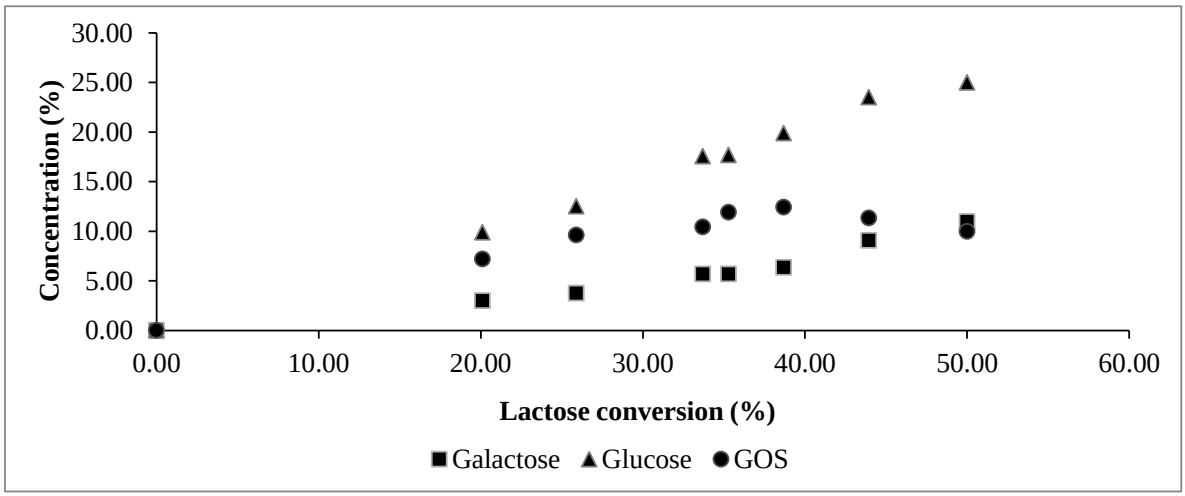
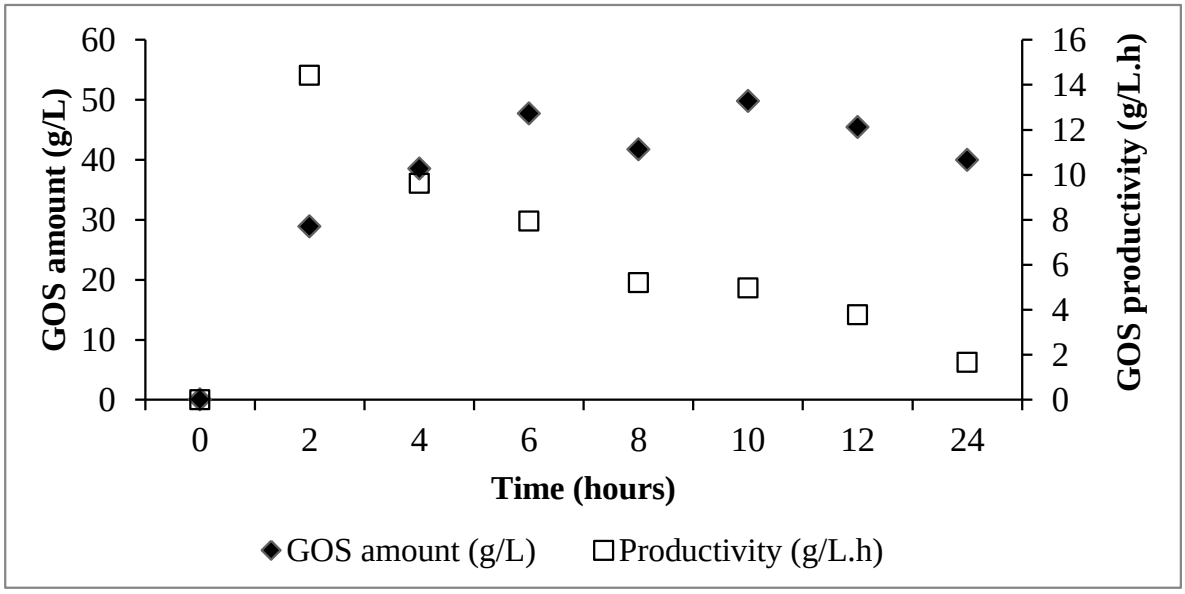


Figure 3: Lactose conversion, and other sugars concentration, catalyzed by immobilized enzyme in the fixed-bed reactor in the first cycle, from 40% (w/w) lactose, pH 5.0, 45ºC. CV% = range of 0.28 to 4.89.

Lactose conversion (%) was defined as residual lactose percent of initial lactose concentration and GOS productivity (g/L.h) as the ratio of total amount of GOS (g/L) produced and reaction time (h).

In the present study a maximum GOS production of 49.72 g/L, after 10 hours of reaction was achieved. Maximum productivity was 14.42 g/L.h after 2 hours of reaction (Figure 4). Total lactose conversion to glucose, galactose and GOS was 44 and 50% after 12 and 24 hours, respectively. Glucose concentration was much higher than galactose which indicates involvement of galactose in GOS formation.



Figur4: Kinetics of GOS synthetized and productivity catalyzed by immobilized enzyme in the fixed-bed reactor in the first cycle, from 40% (w/w) lactose, pH 5.0, 45ºC. CV% = range of 0.48 to 4.50.

Gaur *et al.* (2006) studied *A. oryzae* β-galactosidase covalently coupled to chitosan and aggregated by glutaraldehyde and reported a 17.3 and 4.6% oligosaccharide yield respectively, within 2 h in a 20% (w/v) lactose solution. This corresponds to a productivity of 17 and 4.6 g/L.h for the chitosan immobilized enzyme preparation and cross-linked aggregates, respectively. In another study GOS were continuously produced using *Bullera singularis* β-galactosidase immobilized in chitosan beads in a packed bed reactor with a

productivity of 4.4 g/L.h from 100 g/L lactose and 6.5 g/L.h from 300 g/L lactose solution (SHIN, PARK & YANG, 1998). Pectinex Ultra SP-L, a commercial enzyme preparation obtained from *Aspergillus aculeatus*, containing β-galactosidase activity, was immobilized onto Eupergit C and produced from 30 % (w/v) lactose by 24 h reaction 38.4 and 47.40 g/L of GOS for free and immobilized enzyme, respectively, corresponding to a productivity of 1.6 and 1.97 g/L/h (ASLAN & TANRISEVEN, 2007). β-Galactosidase from *Aspergillus oryzae*, immobilized on glutaraldehyde-treated chitosan beads, produced GOS in a plug reactor and maximum yields were 18, 21, 26% from a lactose solution of 100, 200 and 300 g/L, respectively (SHEU , DUAN & CHEN,1998). Sakai *et al.* (2008) investigated the production of GOS from lactose by alginate-immobilized cells of *Sporobolomyces singularis* in a repeated batch reaction and obtained a maximum GOS yield of 40.4% at a rate of 8.72 g/L.h in a 60% (w/v) lactose solution.

As can be seen in Figure 3, GOS production kinetics is closely related to lactose conversion. As also shown, GOS production increased until a maximum was reached at 10 hours, at 44% lactose conversion and indicate that extending incubation time does not necessarily increase GOS amount. These results suggest that GOS formation precedes lactose hydrolysis as the dominant reaction before this point. Similar patterns were reported by other investigators (NERI *et al*., 2009; SPLECHTNA *et al*., 2007; MATELLA, DOLAN & LEE, 2006; CHOCKCHAISAWASDEE *et al*., 2004; SHEU DUAN & CHEN ,1998).

It is generally observed that the hydrolysis and transgalactosylation reactions occurred simultaneously. What dominates the product profile of the reaction is largely dependent on lactose concentration. β-galactosil groups should have a higher probability of attaching to lactose than water as an acceptor at increasing lactose concentration (ALBAYRAK & YANG, 2002). So the process of GOS formation may be concluded as a

balance between hydrolysis and transgalactosylation (ZHENG *et al.*, 2006). This phenomenon was observed in the study as described above.

It is noteworthy that all types and sizes of GOS including transgalactosylated disaccharides are considered nondigestible oligosaccharides due to similar physiological characteristics, although some differences and strain specificities have also been reported (ALBAYRAK & YANG, 2002).

4. CONCLUSIONS

Since the immobilization efficiency was satisfactory and immobilized enzyme retains its activity without decrease for 30 days, the covalent coupling *A. oryzae* β-galactosidase to chitosan could be used for the production of GOS and to hydrolyze lactose.

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Capítulo 3

IMMOBILIZATION OF FUNGI β-GALACTOSIDASE ON CELITE TO PRODUCE GALACTOOLIGOSACCHARIDES DURING LACTOSE HYDROLYSIS`

Resumo

Imobilização de β-galactosidase fúngica em celite para a produção de galactooligossacarídeos durante a hidrólise da lactose.

Galactooligossacarídeos foram sintetizados, a partir da lactose, em uma coluna encamisada, com β-galactosidase covalentemente imobilizada em celite. As propriedades da enzima imobilizada foram avaliadas e comparadas àquelas da enzima livre. O pH ótimo para a atividade da enzima na forma livre e imobilizada foi de 4,6 e 4,0, respectivamente. A temperatura ótima constatada para a enzima livre foi de 40 °C, sendo 10 °C acima para a enzima imobilizada. A enzima β-galactosidase imobilizada apresentou alta estabilidade quando estocada em tampão a 4° C por 270 dias e demonstrou boa estabilidade operacional quando utlizada em dez repetições. Os oligossacarídeos foram obtidos, através da coluna, com produtividade de 3,5 g/L.h, a partir de uma solução de 400 g/L (p/v) de lactose, em 12 horas. A lactose foi 84,74% hidrolisada em 24 horas.

Palavras-chaves: β-galactosidase; prebiótico; reação de transgalactosilação; celite, ligação covalente.

IMMOBILIZATION OF FUNGI β-GALACTOSIDASE ON CELITE TO PRODUCE GALACTOOLIGOSACCHARIDES DURING LACTOSE HYDROLYSIS

ABSTRACT

Galactooligosaccharides were synthetized from lactose in a fixed-bed reactor using β-galactosidase from *Aspergillus oryzae* covalently coupled to celite. Properties of immobilized β-galactosidase were characterized and compared with those of the soluble enzyme. The optimum pHs for soluble and immobilized β-galactosidase's activity were 4.6 and 4.0, respectively. The optimum temperature for the free enzyme was 40 °C, yet this value was 10 °C higher when characterizing the immobilized enzyme. Immobilized β-galactosidase had a high stability when stored in buffer at 4 °C for 270 days and had a good operational stability when used 10 times repeatedly. Oligosaccharides were obtained in fixed-bed reactor with a productivity of 3.5 g/L.h from 400 g/L (w/v) lactose solution for 12 hours and lactose was 84.74% hydrolyzed for 24 hours.

Keywords: β-galactosidase; prebiotic; transgalactosylation reaction; celite; covalent binding; semicontinuous process.

1. INTRODUCTION

Functional foods are generally defined as foods or food ingredients which impart a health benefit above and beyond the nutritional value expected from food (GOSLIN *et al*. 2010). The group of foods that contains galactooligosaccharides (GOS) is an example of

functional foods, which, after *in vitro* animal and human studies, have been established as prebiotic ingredients (TORRES *et al*., 2010).

GOS have many beneficial effects, such as improving lactose tolerance and digestibility of milk products; preventing pathogenic, autogenic diarrhea and constipation; increasing absorptions of different minerals in the intestine; reducing toxic metabolites, undesirable enzymes and serum cholesterol; depressing blood pressure, among others. Therefore, their application as a food additive for health purposes has led to an increase in their commercial demand (ZHENG *et al*., 2006).

Moreover, the stability under acidic conditions of GOS during food processing makes them potentially applicable as ingredients for a wide variety of food products. Their excellent taste quality and relatively low sweetness make GOS interesting functional sweeteners. They pass the small intestine without being digested and are, therefore, of low caloric value. In addition, GOS cannot be metabolized by microorganisms of the oral cavity and are thus not implicated in the formation of dental caries (MANERA *et al*., 2010; CRITTENDEN & PLAYNE, 1996).

GOS could be produced by *β*-galactosidase (EC.3.2.1.23) treatment of high concentrations of lactose in a transgalactosylation reaction. This transgalactosylation reaction is an intermediary step when galactose units are polymerized to a glucose end unit to form GOS of several polymerization degrees. However, as the reaction continues, all sugars, including GOS, will be hydrolyzed to glucose and galactose monosaccharides (MATELLA, DOLAN & LEE, 2006).

β-Galactosidases are present in a wide variety of sources including plants, animals and microorganisms. As for most reactions catalysed by a biological entity, there are many modes in which GOS producing reactions can be performed using free or immobilized

enzymes, or even whole cells (GOSLIN *et al*., 2010; PANESAR, KUMARI & PANESAR, 2010).

The use of immobilization technology is of significant importance from an economic point of view since it makes reutilization of the enzyme as well as continuous operation possible and can also help to improve the enzyme stability.

The classification of the immobilization methods is related to the properties of the original enzyme, the type of support used and the methods of support activation as well as enzyme attachment (WORSFOLD, 1995). Adsorption and covalent coupling are two of the most used techniques, each one with their own merits and demerits. Adsorption is simple but causes problems of leaching; covalent coupling overcomes this, but harsh conditions lead to undesirable loss of activity at times (WORSFOLD, 1995; PANESAR, KUMARI & PANESAR, 2010).

Some previous research has shown a promising potential to synthesis GOS by different types of *β*-galactosidase immobilization techniques and undoubtedly these investigations are important steps in commercial and fundamental enzymology (MATELLA, DOLAN & LEE, 2006; GAUR *et al*., 2006; LI *et al*., 2008; GROSOVÁ, ROSENBERG & REBROS, 2008). The present study demonstrates an appropriate immobilization method for β-galactosidase on celite, which can be applicable in oligosaccharide synthesis in fixed bed reactors.

2. MATERIALS AND METHODS

2.1 Material

Aspergillus oryzae β-galactosidase (EC 3.2.1.23) and (p-aminophenyl) trimethoxysilane were obtained from Sigma Co., USA. A glucose oxidase kit was purchased from Labtest, Brazil. All the other reagents used were of analytical grade.

2.2 Enzyme adsorption on celite support

2 mg of β-galactosidase (23.96 U/mg) were mixed with celite (1 g, suspended in 10 mL of 0.1 M acetate buffer, pH 4.5) and incubated overnight at 20ºC under constant shaking. The preparation was repeatedly washed with buffer until no protein and enzyme activity were detected in the wash, when analyzed as described in sections 2.5 and 2.6. The celite-bound enzyme was suspended in 5 mL of the same buffer and used as an immobilized preparation for further studies (GAUR *et al*., 2006).

2.3 Immobilization support preparation for covalent binding

Celite was heated at 370 ºC in an oven for 3h to remove organic matter (the oven was heated to the temperature, before the celite was treated), followed by silanization with a 0.5% (v/v) (p-aminophenyl) trimethoxysilane (APTS) aqueous solution at pH 3.3. The support was then incubated at 75º C/3h, further washed thoroughly with deionized water and dried at 105 ºC/15 h. The silanized support was then activated with glutaraldehyde 2.5% (v/v) in buffer acetate 0.2 M, pH 5.0 for 45 minutes. The support was exhaustively washed with deionized water, which was followed immediately by the

immobilization procedure. This methodology was adapted from Melo *et al*. (2005) and Zanin & Moraes (1998).

2.4 Celite covalent binding enzyme

β-galactosidase (2 mg) was mixed with celite (1.0 g suspended in 10 mL of 0.1 M acetate buffer, pH 4.5) and incubated overnight at 20 ºC under constant shaking. The preparation was repeatedly washed with buffer until the wastewater was protein and enzyme activity free. The celite-bound enzyme was suspended in 5 mL of the same buffer and used as an immobilized preparation for further studies (GAUR *et al*., 2006).

2.5 Protein estimation

Protein was determined by the Bradford method (1976) using bovine serum albumin (BSA) as the protein standard (GAUR *et al*., 2006).

2.6 β -Galactosidase assay

Activity of free and immobilized β-galactosidase was estimated by the Food Chemical Codex method (FOOD CHEMICAL CODEX, 1991), using o-nitrophenyl-β D-galactopyranoside (ONPG) as the substrate. Enzyme activity was defined as the amount of enzyme that liberated 1 µmol of o-nitrophenol (ONP) per minute under the standard assay conditions. An extinction coefficient for ONP of 4.3 mM was calculated and used.

2.7 Effect of pH and temperature on enzyme activity

Optimum temperature and pH were determined by individually changing the conditions of the β-galactosidase activity assay: pH from 4 to 9 and temperature from 30 to 70 ºC. An acetate buffer solution (0.2 M) was used for solutions at pH values between 4

and 6. Phosphate buffers (0.2 M) were used for values above this range (TANRISEVEN & DOGAN, 2002).

2.8 Determination of kinetic parameters Km and Vmax

Free and immobilized β-galactosidase were used to measure the kinetic using ONPG as the substrate. Km and Vmax were determined using the Lineweaver–Burk plot method (ZHOU & CHEN, 2001).

2.9 Synthesis of galactooligosaccharides

Fixed-bed reactor studies were carried out in a jacketed glass column with lactose recycle in order to produce galactooligosaccharides. The immobilized β-galactosidase, obtained as previously described, was transferred to a jacketed column (2×8 cm) and to 40% lactose solution in 0.2 M acetate buffer (w/v), pH 4. This lactose solution circulated through a fixed-bed reactor in a recycle process using a MasterFlex® L/S peristaltic pump. Temperature of the column was maintained at 45 °C using an ultrathermostate Quimis® Q-14 M2 bath (precision temperature ±0.1°C). Sample aliquots (1 mL) were collected at various times from 2 to 24 hours and kept in a boiling water bath for 10 min. Oligosaccharides formed were analyzed as described in section 2.10.

2.10 Estimation of oligosaccharides

The identification and quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD). A DIONEX (USA) chromatograph, supplied with a Carbopac PA1 (4x250 mm) column, a PA1 (4x50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and PEAKNET software (DIONEX , USA) were used for the

analyses. Sugars were eluted with 20 mM sodium hydroxide, at a flow rate of 1.0 mL/min at room temperature. Before injection, the samples were diluted with water and filtered through 0.22 µm filters (MANERA *et al*., 2010).

3. RESULTS AND DISCUSSION

β-galactosidase adsorbed on celite and activated with glutaraldehyde retained nearly 11.3 and 27% of the original activity, respectively. As it can be observed, enzymatic activity of covalent binding immobilization in this study was 2.5 fold higher when compared to the adsorption technique used. For this reason, we chose to continue this study using only the covalent immobilization technique. Table 1 compares the results of free and celite covalent binding enzyme in terms of enzymatic activity and kinetic parameters.

Table 1: Characterization of free and immobilized *Aspergillus oryzae* β-galactosidase. Values represent the means of triplicate sets.

Parameters	**Free enzyme**	**Covalent binding enzyme**
Enzymatic activity (U/mg)	11.98 ± 1.73	3.23 ± 0.65
pH optima	4.6 ± 0.00	4.0 ± 0.00
Temperature optima (°C)	40 ± 0.00	50 ± 0.00
Km (mM)	2.34 ± 0.49	9.12 ± 1.66
Vmax (µmol ONP/min)	43.47 ± 1.54	15.08 ± 0.29

It is interesting that Vmax was less affected than Km upon immobilization in the present study. The change in affinity between the enzyme and its substrate is caused by lower affinity between the substrate and the active site of the immobilized enzyme (HAIDER & HUSAIN, 2009). Due to the covalent binding, the Km value increased when compared to soluble β-galactosidase. The change of Vmax points to conformational changes in the enzyme during immobilization.

One of the limitations associated with enzyme industrial application is their high cost and instability under operational conditions. The overall process becomes cost-effective if the preparation shows higher efficiency, operational stability and reusability (GAUR *et al*., 2006). Celite covalent binding enzyme, in the present research, was reused for 10 cycles without significant loss in the enzymatic activity (retained at least 86% of its activity) and after 270 days of storage, suspended in 0.1 M acetate buffer, pH 4.5 at 3.5 °C, retained 85% of its residual enzymatic activity.

For kinetic parameters, pH, thermal, storage, and operational stability, both free and immobilized enzyme were determined using ONPG. It is assumed that both reactions (synthesis and hydrolyze) are usually the same for these enzymatic parameters (HUBER, KURZ & WALLENFELS, 1976; BECERRA *et al*., 2001).

It is worthy highlighting that glycosidases, in general, are known for their capability to carry out hydrolytic catalysis and transfer reactions, where the sugar residue forming the glucone part of the substrate can be transferred to water or any other hydroxyl acceptor sugars groups. It has been established that the same enzyme catalyzes hydrolytic, synthetic and transfer reactions when it comes to β-galactosidase. These observations can be explained assuming that the acceptor and aglycone occupy the same position in the enzyme molecule (WALLENFELS & MALHOTRA, 1961).

It is precisely for these reasons that β-galactosidase has received particular interest since it can mediate the transgalactosylation reaction for the preparation of GOS, just as hydrolytic reaction of lactose for the preparation of low-lactose milk and dairy products.

The GOS production and lactose hydrolysis was monitored and is shown in Figure 1 and Figure 2, respectively.

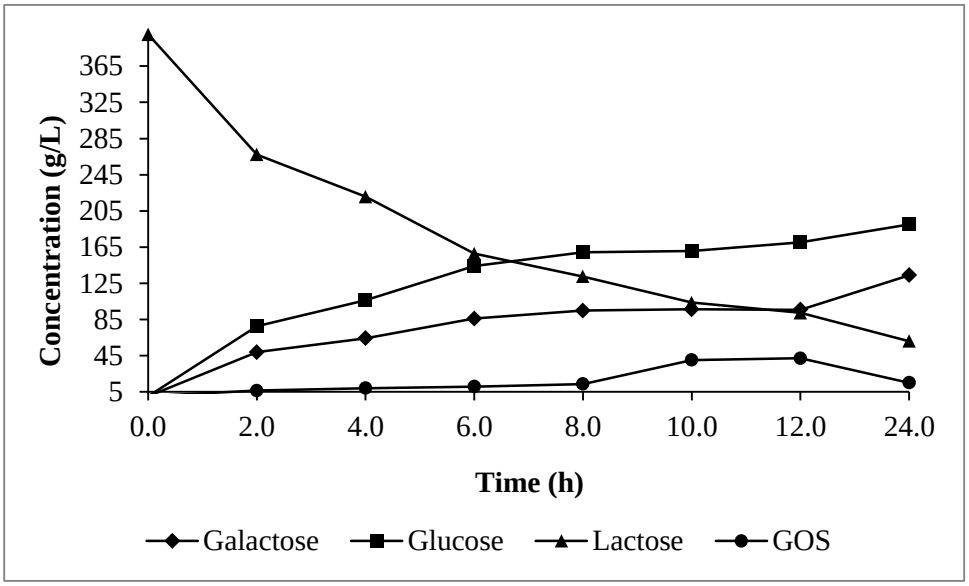

Figure 1: Time course of GOS synthesis in the fixed-bed reactor with lactose recycle in the first cycle, from 40% (w/w) lactose, pH 5.0, 45ºC. CV% = range of 0.44 to 4.80.

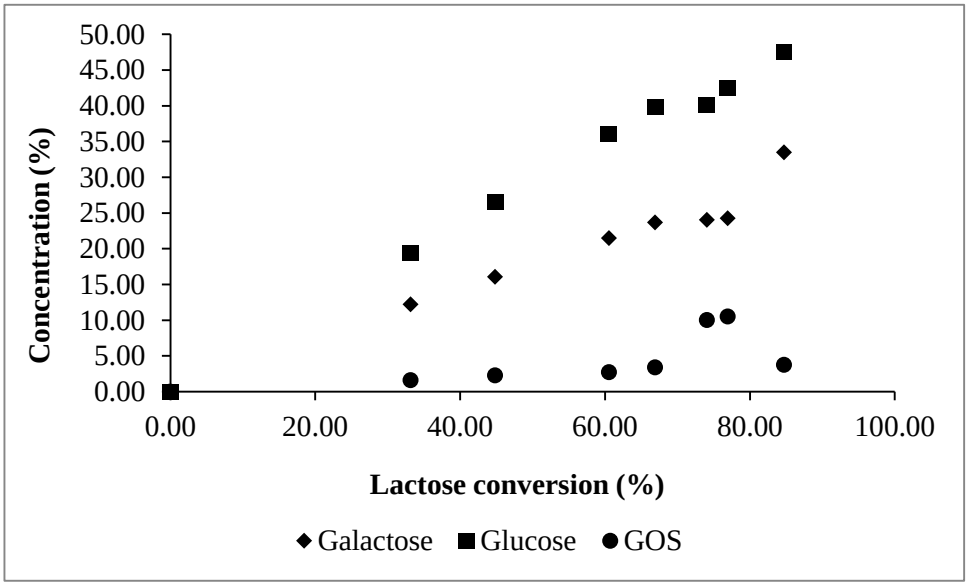

Figure 2: Lactose conversion, and other sugars concentration, catalyzed by immobilized enzyme in the fixed-bed reactor in the first cycle, from 40% (w/w) lactose, pH 5.0, 45ºC. CV% = range of 0.44 to 4.77.

In the present study a maximum GOS production of 42 g/L, after 12 hours of reaction, showing a productivity of 3.5 g/L.h as seen in Figure 3.

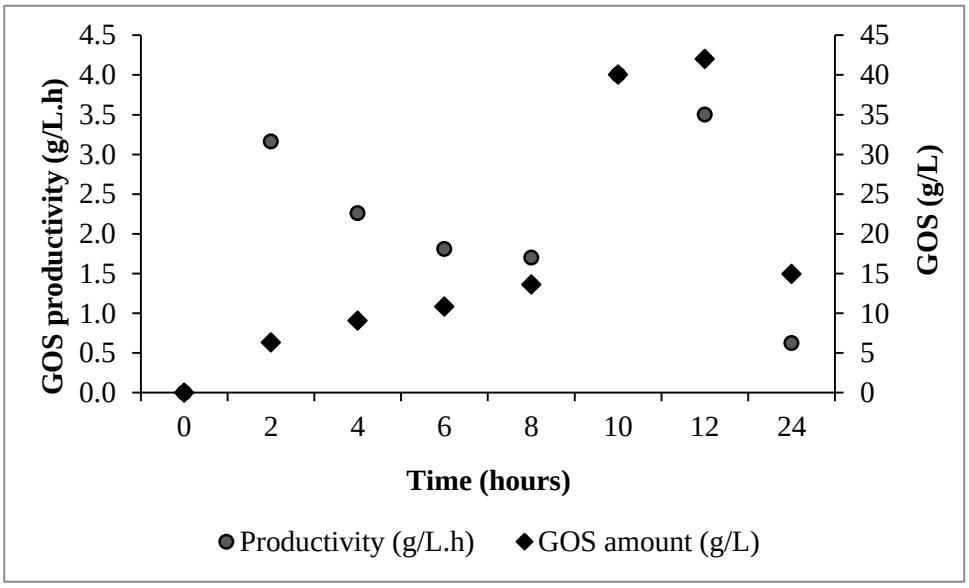

Figure 3: Kinetics of GOS synthetized and productivity catalyzed by immobilized enzyme in the fixed-bed reactor in the first cycle, from 40% (w/w) lactose, pH 5.0, 45ºC. CV% = range of 0.44 to 4.60.

The maximum productivity of GOS obtained under these conditions was 4.0 g/L.h after 10h. The total lactose conversion to glucose, galactose and GOS were 76.93 and 84.74% after 12 and 24 hours, respectively. Packed bed reactor was reused for 10 cycles.

Our results also indicate that extending incubation time does not necessarily increase GOS amount as seen in Figure 3. This result is mainly due to the GOS formation as an intermediary step of β-galactosidase hydrolysis, which is dominant near the beginning of the reaction (MATELLA, DOLAN & LEE, 2006).

The mechanism outlined for β-galactosidase indicates that the enzyme will transfer galactose to nucleophilic acceptors containing a hydroxyl group. If the transference happens with water, galactose will happen as the reaction product; if it happens with

another sugar, di-, tri- and higher galactosyl-saccharides, collectively termed oligosaccharides, will be obtained instead. In turn, these products happen to be the substrates for the enzyme, being slowly hydrolyzed (MAHONEY, 1998).

These results concur with numerous researchers that have also synthesized GOS using immobilized β-galactosidases from different microorganisms (ALBAYRAK & YANG, 2002; CHOCKCHAISAWASDEE *et al*., 2005; GAUR *et al*., 2006; SPLECHTNA, NGUYEN & HALTRICH, 2007; MANERA *et al*., 2010).

Pectinex Ultra SP-L, a commercial enzyme preparation obtained from *Aspergillus aculeatus*, containing β-galactosidase activity, was immobilized onto Eupergit C and produced from 30 % (w/v) lactose by 24 h reaction 38.4 and 47.40 g/L of GOS for free and immobilized enzyme, respectively, corresponding to a productivity of 1.6 and 1.97 g/L/h (ASLAN & TANRISEVEN, 2007).

Gaur *et al*. (2006) studied *A. oryzae* β-galactosidase covalently coupled to chitosan and aggregated by glutaraldehyde and reported a 17.3 and 4.6% oligosaccharide yield respectively, within 2 h in a 20% (w/v) lactose solution. In their work the maximum oligosaccharide productivity was 17 and 4.6 g/L.h for the chitosan immobilized enzyme preparation and cross-linked aggregates, respectively.

In another study GOS were continuously produced using *Bullera singularis* β-galactosidase immobilized in chitosan beads in a packed bed reactor with a productivity of 4.4 g/L.h from 100 g/L lactose and 6.5 g/L.h from 300 g/L lactose solution (SHIN, PARK & YANG, 1998).

4. CONCLUSIONS

The production of GOS using *Aspergillus oryzae* β-galactosidase covalently coupled to celite was described in this paper. Immobilized β-galactosidase had high stability and good operational stability, which points to its excellent potential to be used as support. Oligosaccharides obtained in fixed-bed reactor reach a maximum productivity of 4.0 g/L.h from 400 g/L (w/v) lactose solution in 12 hours. The maximum 42 g/L-GOS-production, was achieved after 12 hours of reaction and the total lactose conversion to glucose, galactose and GOS were 76.93 and 84.74% after 12 and 24 hours, respectively. Thus, the process can be an interesting alternative for GOS production and lactose hydrolyze.

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Capítulo 4

SYNTHESIS OF GALACTOOLIGOSACCHARIDES FROM LACTOSE BY

Pseudozyma tsukubaensis* AND *Pichia kluyveri

Resumo

Síntese de galactooligossacarídeos a partir da lactose por *Pseudozyma tsukubaensis* e *Pichia kluyveri*.

As leveduras vêm sendo utilizadas para a síntese de bio-substâncias de interesse há bastante tempo, com uma vasta gama de culturas exibindo os mais variados benefícios e aplicações à biotecnologia clássica e moderna. *Pichia kluyveri* e *Pseudozyma tsukubaensis* foram isoladas de pêssego (*Prunus persica*) e nectarina (*Prunus persica* var. *nucipersica*), respectivamente, e identificadas através de metodologia molecular (D1/D2 28S rDNA) e análise filogenética. As leveduras isoladas foram testadas quanto à capacidade para produção de galactooligossacarídeos, a partir da lactose, utilizando células viáveis. Foram realizadas, ainda, análises *in vitro* para se averiguar possíveis características probióticas de ambas leveduras. Um rendimento máximo de 14,01 e 15,71% de galactooligossacarídeos foi obtido a partir de 40% (p/v) de lactose por *P. kluyveri* e *P. tsukubaensis*, respectivamente, a 30 °C, pH 7,0 por 24 h. Até onde sabemos, este é o primeiro estudo a reportar o potencial de *P. kluyveri* e *P. tsukubaensis* para a produção de galactooligossacarídeos a partir da lactose. A avaliação dos atributos probióticos revelaram que a cepa de *P. kluyveri* apresenta potencial probiótico em relação à termotolerância, atividade antibacteriana frente à algumas bactérias patogênicas, bem como tolerância a meio ácido e bile.

Palavras-chaves: Endofítico; transgalactosilação; prebiótico; probiótico.

SYNTHESIS OF GALACTOOLIGOSACCHARIDES FROM LACTOSE BY *Pseudozyma tsukubaensis* AND *Pichia kluyveri*

ABSTRACT

Yeasts have been in use for the synthesis of interesting biosubstances for a very long time with a number of new cultures exhibiting a variety of benefits and applications to modern and classical biotechnology. *Pichia kluyveri* and *Pseudozyma tsukubaensis* were isolated from peach (*Prunus persica*) and nectarine (*Prunus persica* var. *nucipersica*), respectively and identified by using molecular approach (D1/D2 28S rDNA) and phylogenetic analysis. The isolated yeasts were tested for their ability to produce galactooligosaccharides from lactose using living whole cells. An *in vitro* analysis of probiotic properties of both yeasts was also carried out. A maximum yield of 14.01 and 15.71% (w/w) galactooligosaccharides was obtained from the reaction from lactose solution (40% w/v) by *P. kluyveri* and *P. tsukubaensis*, respectively at 30°C, pH 7.0 for 24 h. For the best of our knowledge this is the first study reporting the potential of *P. kluyveri* and *P. tsukubaensis* to produce galactooligosaccharides from lactose. Evaluation of their probiotic attributes revealed that only *P. kluyveri* strain has desirable probiotic potential with reference to its thermotolerance, antibacterial activity against some pathogenic bacterial species and tolerance to acid and bile.

Keywords: Endophyte, transgalactosylation, prebiotic, probiotic.

1. INTRODUCTION

Besides being important in the fermentation of foods and beverages, yeasts have shown numerous beneficial effects on human health and potential uses in biotechnology. Thus, there seems to be a need to understand the positive effects of yeasts, their mechanisms and employment of them (MOSLEHI-JENABIAN, PEDERSEN & JESPERSEN, 2010).

Among many uses in different fields it has been found that some yeasts strains are suitable to produce galactooligosaccharides (GOS) from lactose. Although GOS synthesized by *Kluyveromyces* genus (MANERA *et al*., 2010; MARTÍNEZ-VILLALUENGA *et al*., 2008; KIM *et al*., 2001;) have been extensively investigated very few studies exist on the transgalactosylation ability by other yeast genera (CHO, SHIN & BUCKE, 2003; ONISHI KIRA & YOKOZEKI, 1996; ONISHI YAMASHIRO, YOKOZEKI, 1995).

The prebiotic condition of GOS is supported by strong scientific evidence (HUERTA, 2011). They can beneficially affect the host by stimulating growth of selected members of the intestinal microflora, reducing, thus, the formation of toxic metabolites, and producing short chain fatty acids (TORRES, 2010). GOS are now used as low calorie sweetener, food ingredients, pharmaceuticals and other biologically active compounds (NAKKHARAT *et al*., 2006; ASLAN & TANRISEVEN, 2007).

As for most reactions catalyzed by a biological entity, there are many modes in which GOS producing reactions can be performed. Using resting or living whole cells removes the need for enzyme isolation which can be demanding and costly. (GOSLIN *et al*., 2010).

Rastall (2004) suggests that GOS manufacturing technology can be modified to utilize enzymes from probiotic strains on the assumption that the resultant products might have enhanced selectivity for the producing organism.

Some yeasts are currently included as probiotics in commercial products, however this is a relatively unexplored area of research, as most of the efforts have been directed to the characterization of the probiotic potential of lactic acid bacteria and bifidobacteria (TIAGO *et al*., 2009).

This work was thus aimed at the screening of available microorganisms for the production of GOS from lactose. *In vitro* assessment of probiotic potential including thermotolerance, ability to tolerate bile, survival in acidic buffer and antimicrobial activity of selected yeasts from fruits and one standard strain (*Saccharomyces boularddi*) was also carried out.

2. MATERIALS AND METHODS

2.1 Isolation and cultivation

Healthy peaches (*Prunus persica*), nectarines (*Prunus persica* var. *nucipersica*) and plums (*Prunus domestica)* were bought at a local grocer, taken to the laboratory and processed within 4 h. Fruits were first washed in running water. Then, under aseptic conditions, the material was sterilized by the immersion sequence described by Pereira (1993): 70% ethanol for 1 min, 3% sodium hypochloride (NaOCl) for 4 min, 70% ethanol for 0,5 min and rinsed thoroughly with sterile distilled water. From this washing water 50 μL were used to do the aseptic control in petri dishes.

Each fruit was aseptically peeled and some slices were inoculated into 500 mL flasks containing 150 mL of Sabouraud dextrose broth (DIFCO Laboratories) and were

incubated at 30 °C under shaking for 72 h. The cultures of each inoculum were streaked on Sabouraud agar plates which were incubated at 30 °C for 72 h. Pure cultures were established by picking and transferring individual colonies with distinct morphological characteristics to the same medium agar.

2.2 Selection

One loop of each fungi and yeast isolated was transferred to a 125 mL Erlenmeyer flask containing 45 mL of a medium comprised 40% lactose (w/v), 0.5% of yeast extract (w/v), 0.1% (w/v) of urea in a 200 mM acetate buffer (pH 5.0). The flasks were inoculated in a rotary shaker (30ºC/150 rpm) for 24 h and stopped by boiling for 10 min. Oligosaccharides formed were analyzed by described in 2.5. In succession, all the strains considered GOS producers were evaluated as utilization of lactose as sole carbon source. The selected microorganisms were submitted to CBMAI/CPQBA/UNICAMP at Campinas/SP, where they were identified by using molecular approach (D1/D2 28S rDNA) and phylogenetic analysis. Fresh yeasts colonies (24 h) were also observed in microscope to confirm their identity after Gram coloration.

2.3 Pre-inoculum

A loop of *Pichia kluyveri* and *Pseudozyma tsukubaensis* (yeasts isolated) fresh cells cultured on YEPD (yeast extract 1%, peptone 2%, and dextrose 2%) agar was transferred individually to a 500 mL conical flask containing 180 mL of YEPD broth and maintained at 30°C for 48 h in a rotary shaker incubator at a speed of 150 rpm. The inoculum was adjusted to a $1x10^8$ cell/mL concentration by measuring the optical density at λ= 600 nm.

2.4 Synthesis of galactooligosaccharides

A suspension (5 mL) of the fermented YEPD broth was inoculated into 50 mL of a medium comprising 40% lactose (w/v), 0.5% of yeast extract (w/v), 0.1% (w/v) of urea in a 200 mM potassium phosphate buffer (pH 6.0 and pH 7.0) and cultivated aerobically on a reciprocal shaker. Sample aliquots (1 mL) were and kept in boiling water bath for 10 minutes to stop the reaction, then diluted, and filtered through a 0.45 μm membrane to remove insoluble particles. Oligosaccharides formed were analyzed by described in 2.5. Cellular growth was measured by optical density of the culture at 600 nm and biomass concentrations (g dry weight/L) were determined using a calibration curve. The calibration curve was calculated using dilutions of a biomass suspension thus, a relationship between biomass concentration (g/L) and optical density (600 nm) can be determined.

2.5 Estimation of oligosaccharides

The identification and quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD). A DIONEX (USA) chromatograph, supplied with a Carbopac PA1 (4x250 mm) column, a PA1 (4x50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and PEAKNET software (DIONEX , USA). were used for the analyses. Sugars were eluted with 20 mM sodium hydroxide, at a flow rate of 1.0 mL/min. Before injection, the samples were diluted with water and filtered through 0.22 μm filters (MANERA *et al*., 2010).

2.6 *In vitro* assessment of probiotic potential

The microorganisms used in this study were: *P. kluyveri*, *P.tskubaensis* and *Saccharomyces boulardii* (Floratil®, Merck S.A., Brazil) as a positive control.

Specific growth (μ) rates were measured during cultivation (at different temperatures). Yeast growth was determined spectrophotometrically by following changes in culture absorbance at 600nm. Specific growth rate was calculated according to the formula: $\mu = (\ln x - \ln x_0) / (t-t_0)$, where x and x_0 were absorbances measured at time t and t_0, respectively, according to Grmanová *et al*. (2010). All tests were run in duplicate and averaged.

2.6.1 Thermotolerance in vitro

As a first criterion, yeast were inoculated in YEPD broth and incubated at 37 °C (human body temperature) for a maximum of 96 h, according to Tiago *et al*. (2009). Specific growth rates were measured during cultivation as described above.

2.6.2 Low pH tolerance

The method was adapted from that described by Toit *et al*. (1998). YEPD broth was adjusted to pH 2, 3, 4, 5, 6 and 8, respectively, using 1M HCl or 1 M NaOH. The media (50 mL) were inoculated with 5 mL of an overnight culture and incubated at 37 °C. The absorbance at 600 nm was measured after four hours. Samples of YEPD broth adjusted to pH 2 were collected at 0, 4 and 7 hours and inoculated in YEPD agar for viable-cell counting (CFU/mL).

2.6.3 Bile-salt tolerance

As a third criterion yeasts were compared for their ability to grow in the presence of bile. YEPD broth was supplemented with 0.3% (w/v) of bile salts (Difco) and adjusted to pH 2 with 1M HCl. Tubes were incubated at 37 °C and the absorbance at 600 nm was monitored at six, twelve and twenty four hours (TOIT *et al*., 1998).

2.6.4 Microorganism strains for antimicrobial assays

The tests indicators *Staphylococus aureus* ATCC 6538, *Salmonella choleraescius* ATCC 10708, *Pseudomonas aeruginosa* ATCC 10145, *Escherichia coli* CCT 0547, *Micrococcus luteus* ATCC and *Serratia marcescens* ATCC 1953 were obtained from Bioaromas Laboratory (Faculdade de Engenharia de Alimentos – FEA/UNICAMP) culture collection.

2.6.5 Antagonism in vitro

Antagonistic activity of the yeasts was determined by the double layer diffusion method (TIAGO *et al*., 2009). The tested yeasts were spotted onto the surface of YEPD agar and incubated at 37 °C, for 48 h. Then, the colonies were killed by exposure to chloroform during 30 min. Residual chloroform was allowed to evaporate and the test cultures were overlaid with 2.5 mL of BHI soft agar (0.7%) which had been inoculated with 0.01 mL of a 24 h broth culture of the indicator strain. Plates were then incubated for an additional 24–48 h period, at 30 °C, and evaluated for the presence of growth inhibition zone.

3. RESULTS AND DISCUSSION

Approximately fifteen endophyte species with distinctive colony characters and probably belonging to distinct taxa were isolated from peach, nectarine and plum in this study. From these isolated strains two yeasts, from peach and nectarine, were selected as capable to produce galactooligosaccharides.

The yeasts were identified by using molecular approach (D1/D2 28S rDNA) and phylogenetic analysis. The partial sequence of 28S rDNA D1/D2 region of the yeast sample isolated from peach presented more than 99% similarity to sequences in the same region of the ribosomal operon of different strains of *Pichia kluyvery* deposited in the database (GenBank). In addition, that yeast formed a cluster which was supported by a high bootstrap value of 95% with different strains of *P. kluyveri* (Figure 1). Therefore, the strain was determined to be *P. kluyveri.*

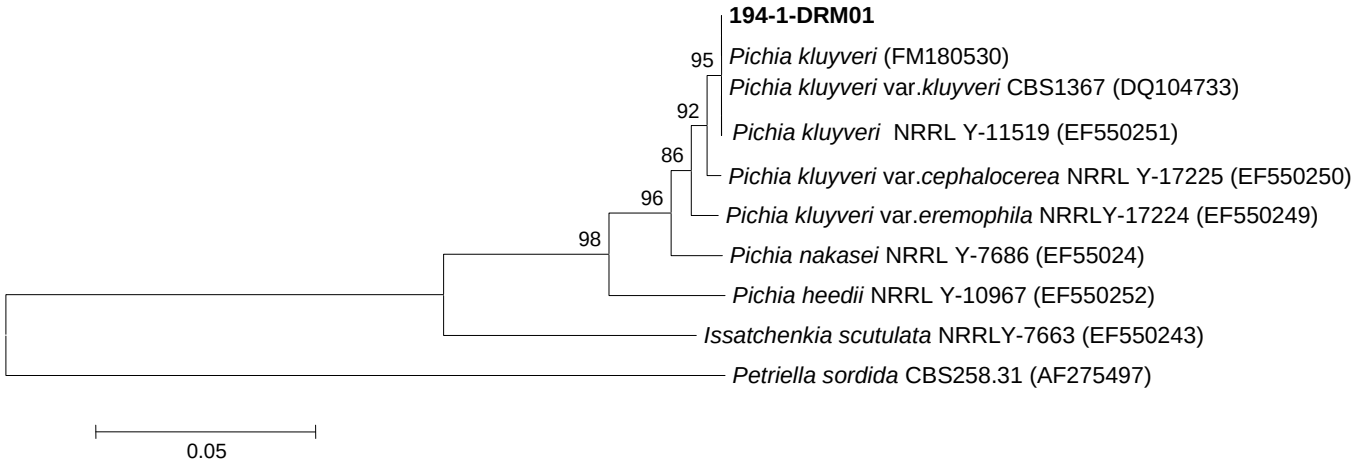

Figure 1: Phylogenetic tree base on 16S rDNA sequences of *P. kluyveri* strain coded as 194-1-DRM0

For the yeast isolated from nectarine the sequence of the D1/D2 region of the ribosome sample *show* a *high* level of *sequence similarity to the* dimorphic fungus *Ustilago spermophora* / *Pseudozyma tsukubaensis* (Figure 2).

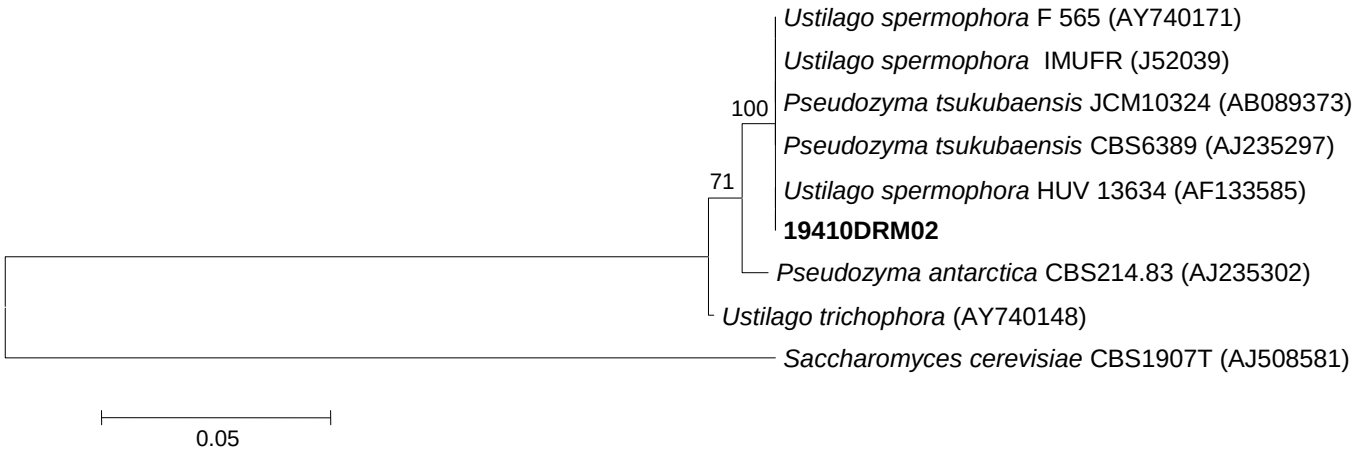

Figure 2: Phylogenetic tree base on 16S rDNA sequences of *P. tsukubaensis* strain coded as 1941DRM02.

This group also forms a clade together with 100% bootstrap support. Hence, the strain was determined to be *P. Tsukubaensis*. It is noteworthy that *P. tsukubaensis* is the yeast phase of *U. spermophora* (Begerow & Bauer, 2000).

Microscope analysis performed also confirmed the identity of both yeasts. This evaluation was based on the morphology of the colony and their characteristics in microscope.

Pichia is a representative yeast genus that have been isolated and recognized as an endophyte in a wide variety of fruits and vegetables (DABHOLE & JOISHE, 2005; FREDLUND *et al*., 2002; MORAIS *et al*., 1995). Restuccia *et al*. (2006) isolated from peach *Pichia fermentans, Pichia kluyveri* and *Candida maltose* for antagonism *in vitro* against postharvest pathogenic molds. Qing & Shiping (2000) isolated from wounds of

peach fruit a new yeast antagonist, *Pichia membranefaciens* and evaluated for its biocontrol capability against *Rhizopus stolonifer* on nectarine fruits.

Yeast metabolism is broadly exploited in many biotechnological processes. Physiological and metabolic properties of *Pichia kluyveri* has been described by some author in the last years, most of them related to studies of biocontrol activity (PELLICCIA *et al*., 2011; MASOUD & KALTOFT, 2006).

The strains belonging to the genus *Pseudozyma* are mainly isolated from plants (MORITA *et al*., 2007; GOLUBEV, PFEIFFER & GOLUBEVA, 2006). Morita *et al*. (2010) isolated *Pseudozyma tsukubaensis* from leaves of *Perilla frutescens* in Japan to produce glycolipid biosurfactant. Mannosylerythritol lipids are abundantly produced from vegetable oils and sugars by the yeast strains of the genus *Pseudozyma*. These glycolipid-type biosurfactants exhibit not only excellent surface-active properties, but also versatile biochemical actions (FUKUOKA *et al*., 2011). In addition, *Pseudozyma* species have attracted great attention of biotechnologists as promising producers of enzymes and recently as biocontrol agents of fungal pathogens of plants (GOLUBEV, 2007; GOLUBEV, PFEIFFER & GOLUBEVA, 2006).

However, as far as we know it has not been previously reported the synthesis of GOS by *P. kluyveri and P. tsukubaensis* until the present study.

The search for "new" sources for oligosaccharide production became necessary as a result of the increasing number of oligosaccharides applications in the cosmetic, agrochemical, pharmaceutical and food industries (MAUGERI & HERNALSTEENS, 2007).

Transgalactosylation activities were investigated for *P. kluyveri* and *P. tsukubaensis* and effects of pH in GOS synthesis were examined for 24 h. For both yeast strains optimal pH was about 7.0 (Figure 3).

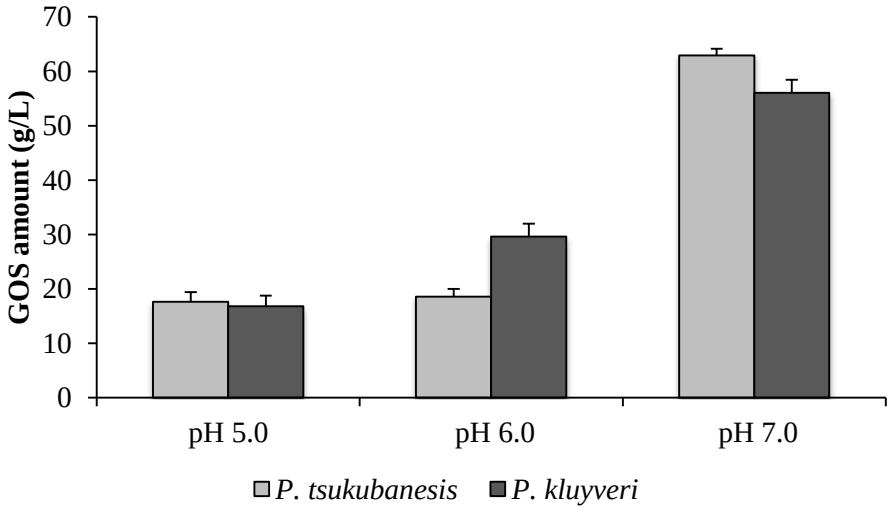

Figure 3: Influence of pH on GOS synthesis by *P.kluyveri* and *P. tsukubaensis* from 40% (w/w) lactose, 30ºC,24 h,150 rpm.

A maximum GOS production yield of 14.01 and 15.71 % (w/w) for *P. kluyveri* and *P. tsukubaensis* cells were observed, respectively (Table 1).

Table 1: Comparison of GOS yield and productivity synthesized by several yeasts.

Yeast	GOS Yield (%)	Productivity (g/L.h)	Reference	GOS production method
P. tsukubaensis	**15.71 ± 0.60**	**2.62 ± 0.10**	**This study**	**Fermentation**
P.kluyveri	**14.01 ± 0.87**	**2.34 ± 0.15**		
Apiotrichum humicola	13.33	2.50	Onishi Yamashiro & Yokozeki, 1995	Fermentation
Cryptococcus laurentii	14.33	2.68		
Geotricum amycelicum	19.00	3.56		
Kluyveromyces fragilis	12.00	2.25		
Sirobasidium magnum	37.7	3.20	Onishi & Yokozeki, 1996	
Kluyveromyces maxianus	25.10	1.39	Kim *et al*., 2001	Permeabilized Cells
Kluyveromyces maxianus	16.70	19.00	Manera *et al*., 2010	

Yield (%) = [GOS (g/L)/ initial lactose concentration]* 100
Productivity (g/L.h) = GOS (g/L)/reaction time (h)

The total amount of GOS produced from 40% (w/w) lactose were 56,04 g/L for *P. kluyveri* and 62.96 g/L for *P. tsukubaensis* at pH 7.0 and 30ºC in 24 hours.

It is worthy highlighting that despite the capacity for GOS synthesis by both yeast cells, the final concentration of galactose in the systems were low as can be seen in Table 2.

Table 2: Comparison of GOS and galactose production by various yeasts.

Yeast	GOS produced (g/L)	Galactose produced (g/L)	Lactose hydrolysis (%)	Reference
P. tsukubaensis	**62.96 ± 1.64**	**12.11 ± 1.89**	**24.00 ± 2.60**	**This study**
P.kluyveri	**56.04 ± 2.27**	**10.26 ± 0.33**	**20.00 ± 0.66**	
Sirobasidium magnum	63.00	6.0	33.00	Onishi Yamashiro & Yokozeki, 1995
Sterigmatomyces elviae	74.00	5.0	30.00	
Rhodotorula minuta	67.00	7.0	40.00	

This result indicates that the capacity of both yeasts strains to perform the transgalactosylation synthesis is greater than the lactose hydrolytic reaction. Similar results were found by Onishi, Yamashiro & Yokozeki (1995) screening numerous yeasts for the ability to produce GOS as seen in Table 2. In addition, both yeasts demonstrated ability to use lactose as sole carbon source at high concentrations of this disaccharide. In other words: *P. kluyveri* and *P. tsukubaensis* cells multiply in the presence of high lactose concentration and not simply stand in a non-proliferation condition while GOS are synthesized.

A continuous increase of biomass in 48 hour of growth in the GOS medium at pH 7.0 was observed for both yeasts. The maximum biomass yield was 6.85 and 5.70 g/L for *P. kluyveri* and *P. tsukubaensis*, respectively obtained after 48 hours.

As one of the largest and most biodiverse countries in the world, Brazil may provide a rich source of microorganisms for potential probiotic use (TIAGO *et al*., 2009). In the present study, GOS producers yeasts isolated from fruits were tested as possible probiotics using *in vitro* assays.

Tolerance to elevated temperature is rarely found in yeast from non-pathogenic sources (TIAGO *et al*., 2009). However, *P. kluyveri* had a good and fast growth at 37 °C, when compared to *S. boulardii*, which was used as reference (Figure 4).

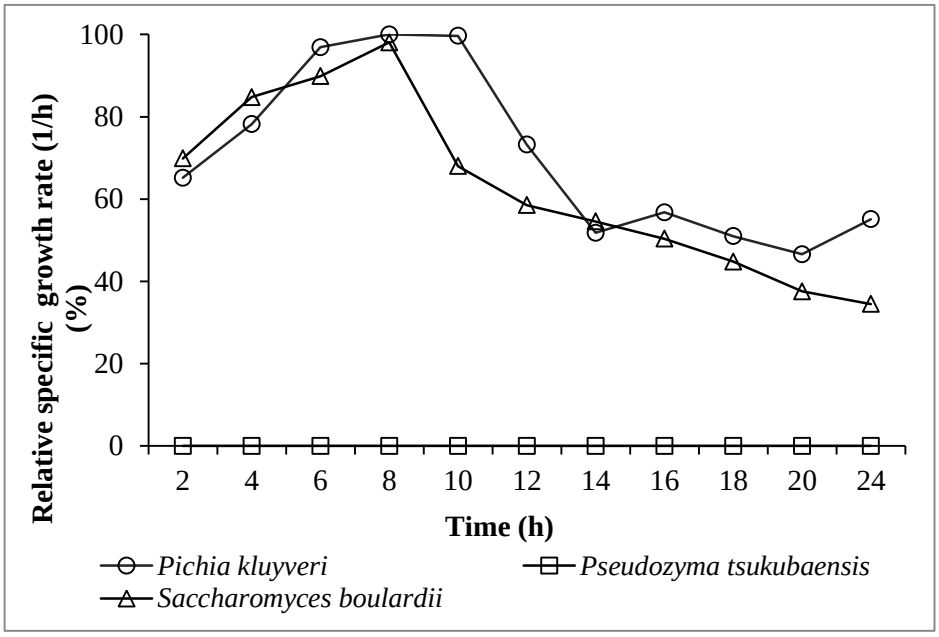

Figure 4: Relative specific growth rates of *P.kluyveri*, *P. tsukubaensis,* and *S. boulardii* growing on YEPD broth at 37°C. CV% = range of 0.69 to 6.45.

Thermotolerance was not observed for *P. tsukubaensis*. Therefore the other *in-vitro*-probiotic-assays presented in this study will only investigate the potential of *P. kluyveri*.

Probiotics microorganisms must confront a number of successive adverse conditions all along the gastrointestinal tract and this difficult is particularly significant when the microorganisms are not originated from the digestive tract of mammals (MARTINS *et al*., 2008), as in the case for the isolates yeast tested in this study.

Each day the human stomach secretes about 3 L gastric juice at a pH of around 2, therefore it is necessary for probiotic microorganisms to be acid-resistant if they are to reach the colon unaided (VERNAZZA, GIBSON, RASTALL, 2006). As can be seen in Figure 5, pH 2 was not lethal for *P. kluyveri*, even after 24h exposure.

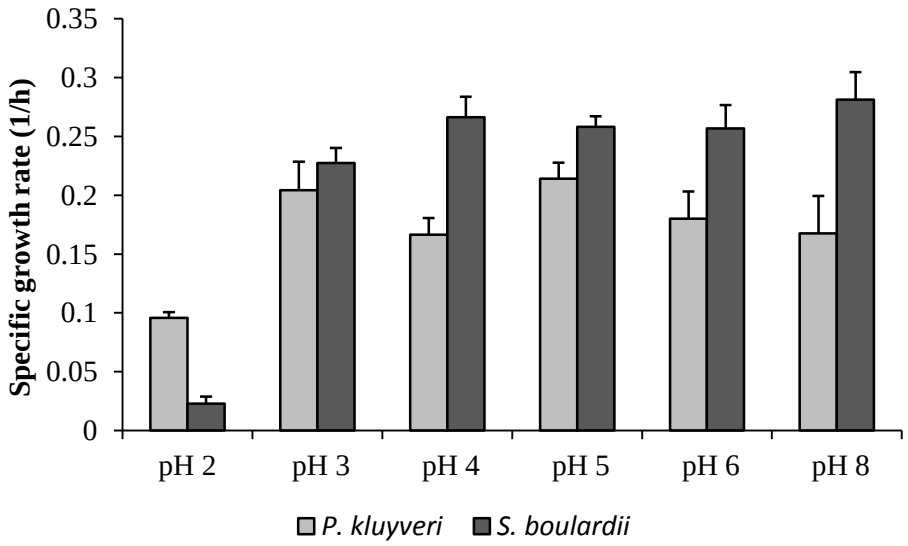

Figure 5: Effect of pH on specific growth rates of *P.kluyveri* and *S. boulardii* growing in YEPD broth at 37°C for four hours.

Regarding the effect of pH 2 on viable cell count the total cultivable cells remained at 6 log CFU/mL throughout the time course. Raising the pH to three or four gave improved specific growth rates as expected. It is worth noting that growth rates were similar for all of the test conditions, indicating that this strain would have an increased chance of passing through the stomach intact. *S. boulardii* specific growth profile was similar to the one observed for *P. kluyveri*.

As well as *S. boulardii*, *P. kluyveri* was able to grow in 0.3% (w/v) bile salts as demonstrated by specific growth rate presented in Figure 6.

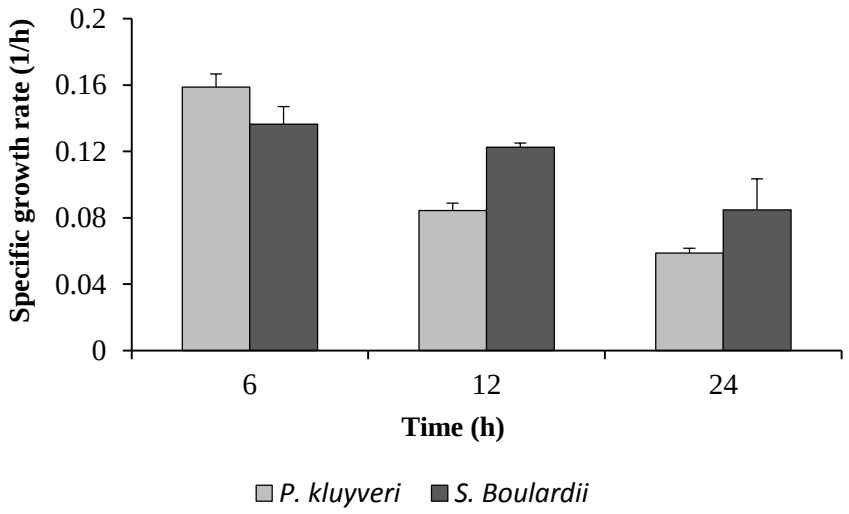

Figure 6: Specific growth rates of *P.kluyveri* and *S. boulardii* growing in YEPD broth supplemented with 0.3% (w/v) of bile salts and adjusted to pH 2 at 37°C.

Agarwal *et al*. (2000), Erkkila & Petaja, (2000) and Jin *et al*. (1998) considered 0.3% bile salts as a critical concentration to screen for resistant strains when selecting human probiotics.

Bile salts are released into the small intestine after ingestion of a fatty meal and its detergent-like function is critical to microorganisms since their cell membranes are composed of lipids and fatty acids. However, some microorganisms are able to hydrolyze bile salts with bile salt hydrolase enzyme (BSH) decreasing their solubility, and thus weakening their detergent effect. BSH activity has been found in many species as *Lactobacillus*, for instance, but the resistance to bile salts varies a lot between the *Lactobacillus* species and even between strains and the mechanism is still unknown (ERKKILA & PETAJA, 2000).

In order to obtain supplementary information, antagonism assays were also performed. *P. kluyveri* and *S. boulardii* exhibited a diversified pattern with respect to antibacterial activity against the five indicator bacterial species as can be seen in Table 3

Table 3: Inhibitory activity of *P.kluyveri* and *S. boulardii* against pathogenic bacterial species.

Indicator strains	Inhibitory activity*	
	P. kluyveri	***S. Boulardii***
Staphylococus aureus ATCC 6538	+	-
Salmonella choleraescius ATCC 10708	-	-
Pseudomonas aeruginosa ATCC 10145	+	+
Escherichia coli CCT 0547	-	-
Micrococcus luteus CCT 2692	-	-
Serratia marcescens ATCC 1953	-	+

* + = Clear inhibition zone of 1 mm or more, - = no inhibition

Similar behavior of this study was reported by Tiago *et al*. (2009) screening among 103 non-*Saccharomyces* yeasts a candidate for probiotic by using *in vitro* and *in vivo* criteria. The authors concluded that *P. kluyveri* strain encoded as 898 showed to be the best candidate for probiotic use among the tested yeast, considering properties as: colonization and survival in the digestive tract, ability of *in vivo* antagonism against enteropathogenic bacterium and protective effect in animals. It is worth highlighting that the authors tested for probiotic potential two strains of *Pichia kluyveri* and the results were different from each other.

The most probable reason for this result is that some features of the same yeast species are strain specific as reported previously by Agarwal *et al*. (2000). These authors tested various strains of *Saccharomyces cerevisiae* for their tolerance to some adverse gastrointestinal conditions *in vitro* and observed differences behavior among the tested strains. Newbold *et al*. (1995) observed that different strains of *S. cerevisiae* differ in their effects on ruminal bacterial numbers *in vitro* and in sheep.

Chen *et al*. (2010) screening for probiotic yeast strains from raw milk selected *Pichia fermentan, Pichia kudriavzevii* and *Yarrowia lipolytica* as potential probiotics to

assimilate cholesterol in the human intestine. Pennacchia *et al*. (2008) isolated acid- and bile-resistant strains directly from food samples and concluded that *Saccharomyces cerevisiae*, *Pichia galeiformis*, *Pichia membranifaciens* and *Pichia manshurica* demonstrated the best probiotic performance.

It is widely recognized that probiotic properties are strictly strain related (PENNACCHIA, 2008; KUHLE, SKOVGAARD & JESPERSEN, 2005). The *P. kluyveri* strain isolated from peach and tested in this study is capable to synthesize GOS, as well as demonstrates probiotic potential by *in vitro* assays. Moreover, in the recent literature there are no reports of human diseases related to the specie *P. kluyveri*.

The magnitude and diversity of the microbial population make the screening of biotherapeutic agents among them a very hard task, thus a simple and efficient method for this selection is needed. *In vivo* assays are time consuming and involve a large quantity of animal groups and for these reasons are used only after the selection of a limited number of probiotic candidates. Hence, *in vitro* studies have been used to evaluate the capacity of a microorganism to survive in the simulated conditions of the digestive tract since this is indispensable for a probiotic to act (MARTINS *et al*., 2008).

Theoretically, any non-pathogenic bacterium, fungus, yeast or protozoan is a possible candidate for probiotic use (TIAGO *et al*., 2009; MARTINS *et al*, 2008).

At present, the principal yeasts used as probiotic belong to the genus *Saccharomyces*. In particular, the strain *S. boulardii* is the only one commercialized with this purpose in human medicine (PENNACCHIA *et al*., 2008).

So far, efforts have been placed on exploiting the probiotic effects of lactic acid bacteria and bifidobacteria, whereas limited emphasis has been placed on the beneficial advantageous properties offered by yeasts as: a diversified enzymatic profile, a versatile

effect on the immune system, they provide protection against pathogenic bacteria and toxic compounds, they appear to be better suited for nutritional enrichment and delivery of bioactive molecules. Besides yeast are much more robust than lactic acid bacteria and bifidobacteria which make them easier to produce and to distribute (MOSLEHI-JENABIAN, PEDERSEN & JESPERSEN, 2010; TIAGO *et al*., 2009; MARTINS *et al*., 2008; PENNACCHIA *et al*., 2008;).

Thus it is encouraged additional efforts exploring the health beneficial effects of yeasts, especially those properties that cannot be replaced by lactic acid bacteria and bifidobacteria (MOSLEHI-JENABIAN, PEDERSEN & JESPERSEN, 2010).

4. CONCLUSION

This research demonstrated for the first time the possibility to use the yeasts *P. tsukubaensis* and *P. kluyveri* to synthesize galactooligosaccharides from lactose. More research has been done to evaluate and optimize this process. It was also observed that *P. kluyveri* strain may be regarded as a potentially probiotic. *In vitro* studies did not support *P. tsukubaensis* classification among possible candidate for probiotic use. On the other hand *P. kluyveri* strain may be regarded as a potentially probiotic. This study represents a preliminary research and to declare *P. kluyveri* as effective probiotic further investigations such as adhesion capability to the intestinal mucosa cells and specific clinical analyses, concerning human health are required. However, the results obtained up to now suggest the good capacity of the selected *P. kluyveri* strain.

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Capítulo 5

OPTIMIZATION OF GALACTOOLIGOSACCHARIDES SYNTHESIS BY

Pseudozyma tsukubaensis and Pichia kluyveri USING EXPERIMENTAL DESIGN

Resumo

Otimização da síntese de galactooligossacarídeos por *Pseudozyma tsukubaensis* e *Pichia kluyveri* utlizando planejamento experimental.

A síntese do prebiótico galactooligossacarídeo, a partir da fermentação da lactose, por *Pseudozyma tsukubaensis* e *Pichia kluyveri* foi investigada. Neste estudo, dois planejamentos experimentais sequenciais foram utilizados a fim de otimizar a produção de galactooligossacarídeo. Primeiramente, um *screening* do tipo Plackett-Burman foi realizado para se avaliar o efeito da lactose, extrato de levedura, uréia e concentração de inóculo, bem como das variáveis pH e rotação, no rendimento de galactooligossacarídeos sintetizado pelas leveduras. O delineamento de Plackett-Burman permitiu identificar a concentração de uréia e o pH como variáveis estatisticamente significativas, na faixa de estudo analisada. Em seguida, para determinar as condições ótimas para a síntese de galactooligossacarídeos foi realizado um delineamento composto central rotacional. As variáveis independentes selecionadas foram concentração de lactose, extrato de levedura e uréia, e a resposta, o rendimento de galactooligossacarídeos prozido por *P. tsukubaensis*. As condições otimizadas para a síntese deste prebiótico foram: lactose, extrato de levedura e uréia nas concentrações de 26,00; 0,80 e 1,80%, respectivamente. Sob as condições otimizadas e validadas, a atividade de transgalactosilação de *P. tsukubaensis* resultou em um rendimento máximo de galactooligossacarídeo de 28,35% em 24 horas, com uma produção de 73,71 g/L e, aproximadamente, 50,00% de hidrólise da lactose.

Palavras-chaves: Lactose; atividade de transgalactosilação; prebiótico; metodologia de superfície de resposta.

OPTIMIZATION OF GALACTOOLIGOSACCHARIDES SYNTHESIS BY *Pseudozyma tsukubaensis* and *Pichia kluyveri* USING EXPERIMENTAL DESIGN

ABSTRACT

Synthesis of the prebiotic galactooligosaccharides from lactose fermentation by *Pseudozyma tsukubaensis* and *Pichia kluyveri* was investigated. In this study, a sequential strategy of two experimental designs was used to optimize galactooligosaccharides production. First, Plackett-Burman screening design was applied to evaluate the effects of lactose, yeast extract, urea and inoculum concentrations, pH and agitation rate on the response of galactooligosaccharides yield synthesized by both yeasts. The preliminary Plackett–Burman design allowed to identify urea concentration and pH as statistically significant variables in the range studied. In order to find the optimal conditions for galactooligosaccharides synthesis, a central composite rotational design was performed. The independent variables were lactose, yeast extract and urea concentrations and the response take into account was galactooligosaccharides yield produced by *P. tsukubaensis*. The optimized condition for this prebiotic synthesis was: lactose, yeast extract and urea at concentrations of 26.0, 0.8 and 1.8%, respectively. Under the optimized and validated conditions the transgalactosylation activity of *P. tsukubaensis* resulted in 28.35% of GOS yield at 24h with a GOS production of 73.71 g/L and approximately 50% of lactose hydrolysis.

Keywords: Lactose; transgalactosylation; prebiotic; response surface methodology

1. INTRODUCTION

There is a variety of non-digestible oligosaccharides produced worldwide, intended for use as functional food ingredients (GOULAS, TZORTZIS & GIBSON, 2007). Amongst these, galactooligosaccharides (GOS), which are generally recognized as safe, have now been definitely established as prebiotic ingredients. Thus, the production of this prebiotic has become commercially important (PARK & OH, 2010; TORRES *et al*., 2010).

For this reason, ingested GOS are now wildly used as a foodstuff valuable additives, as the products of fermentation of GOS in the colon, mainly short chain fatty acids, have a role in the improvement of the colonic environment, energy supply to the colonic epithelium, and calcium and magnesium absorption (PETROVA & KUJUMDZIEVA, 2010; MACFARLANE, STEED & MACFARLANE, 2007; SAKO, MATSUMOTO & TANAKA, 1999).

Goslin *et al*. (2010) and Torres *et al*. (2010) review the progress, impact and advances made during the last decade in the biotechnological production of GOS, as well as their commercial applications.

GOS are principally synthesized by enzymatic transgalactosylation reaction from lactose by β-galactosidases from various microorganisms, obtaining complex mixtures of oligosaccharides with different glycosidic linkages and degree of polymerization (CARDELLE-COBAS *et al*., 2011; SANTOS, SIMIQUELI & PASTORE, 2009; PANESAR *et al*., 2006).

It is known that some yeasts exhibits transgalactosylation activity, and efficient GOS synthesis by *Sporobolomyces singularis* (ISHIKAWA *et al*., 2005), *Kluyveromyces marxianus* (MANERA *et al*., 2010), *Bullera singularis* (CHO, SHIN & BUCKE, 2003), *Candida bombicola*, *Dekkera anomala*, *Shizosaccharomyces servazzii* (PETROVA &

KUJUMDZIEVA, 2010), *Apiotrichum humicola*, *Cryptococcus laurentii*, *Kluyveromyces fragilis* (ONISHI, YAMASHIRO & YOKOZEKI, 1995), *Rhodotorula minuta*, *Sirobasidium magnum*, *Sterigmatomyces elviae* (ONISHI & YOKOZEKI, 1996), among others, has been reported in the literature.

The structure and product ratio of GOS obtained by transgalactosylation reactions depend on the biocatalyst as well as on the conditions employed in the biotransformation reaction (HELLEROVÁ & CURDA, 2009; NAKKHARAT *et al*., 2006; BOON, JANSSEN & RIET, 2000).

As for most reactions catalyzed by a biological entity, there are various manners in which GOS production can be accomplished. GOS can be produced either by enzymes or by fermentation and lactose serves both as a galactosyl donor and an acceptor to yield di-, tri-, or higher oligosaccharides (BICAS *et al*., 2010; GOSLIN *et al*., 2010; KIM, LEE & LEE, 2001).

Although many microbial products are synthesized by extracellular means it is suggested that much larger proportion of the potentially useful microbial products is retained within the cells (PANESAR, 2008). Using resting cells or living microorganisms have advantages over purified enzymes in many industrial bioconversion processes since removes the need for enzyme isolation which can be demanding and costly (GOSLIN *et al*., 2010; LEE, KIM & OH, 2004).

Despite considerable work has been done in the last years to improve GOS synthesis process, few studied focused on the alternative to produce GOS by fermentation. Thus, the objective of this paper was to optimize the production of GOS by *Pseudozyma tsukubaensis* and *Pichia kluyveri* fermentation of lactose by using experimental design and surface analysis methodology.

2. MATERIALS AND METHODS

2.1 Microorganisms

The yeasts strains used in this study were previously isolated from health nectarine (*Prunus persica* var. *nucipersica*) and peach (*Prunus persica*) and identified by 16S-rRNA cDNA sequence as *Pseudozyma tsukubaensis* and *Pichia kluyveri*, respectively (not yet published).

2.2 Pre-inoculum

A loop of *Pichia kluyveri* and *Pseudozyma tsukubaensis* fresh cells cultured on YEPD (yeast extract 1%, peptone 2%, and dextrose 2%) agar was transferred individually to a 500 mL conical flask containing 180 mL of YEPD broth and maintained at 30°C for 48 h in a rotary shaker incubator at a speed of 150 rpm. The inoculum was adjusted to a $1x10^8$ cell/mL concentration by measuring the optical density at λ= 600 nm.

2.3 Sequential strategy of experimental design for GOS production

A suspension of the fermented YEPD broth was inoculated into 50 mL of a medium comprising lactose, yeast extract and urea in concentrations and pH defined by the experimental design. All experiments were carried out in Erlenmeyer flasks and cultivated aerobically on a reciprocal shaker, according to the experimental outlined. Sample aliquots (1 mL) were collected at 24, 48, and 72 hour intervals and kept in boiling water bath for 10 minutes to stop the reaction, then diluted, and filtered through a 0.45 µm membrane to remove insoluble particles. Oligosaccharides formed were analyzed by described in 2.4. A sequential strategy of two experimental designs was used to optimize the GOS production. First, Plackett-Burman screening design including 15 trials with three central points (RODRIGUES & IEMMA, 2005) was applied to evaluate the effects of lactose, yeast

extract, urea and inoculum concentrations, pH and agitation rate (independent variables) on the response of GOS yield synthesized by *P. tsukubaensis* and *P. kluyveri*. The preliminary Plackett–Burman design allowed to identify the statistically significant variables, to verify if the investigated levels were in adequate range and to select them to realize a complete factorial design with respect to GOS yield. In order to find the optimal conditions for GOS synthesis, a central composite rotational design CCRD for three independent variables with three replicates at the central point and four axial points (17 trials) was performed (RODRIGUES & IEMMA, 2005). The independent variables were lactose, yeast extract and urea concentrations and the response take into account was GOS yield produced by *P. tsukubaensis*. The statistical design and the coded and real values of the variables for both experimental designs are given in Table 1. The software Statistica 7.0 (Statsoft Inc., USA) was used to analyze the results.

Table 1: Values of coded levels used in Plackett-Burman (design 1) and CCRD (design 2).

	Coded variable level	**Lactose% (w/v)**	**Yeast extract% (w/v)**	**Urea% (w/v)**	**pH**	**Inoculum % (v/v)**	**Agitation rate (rpm)**
Plackett-Burman	-1	30.00	0.20	0.20	4	5.00	100
	0	40.00	1.20	0.40	6	10.00	150
	+1	50.00	2.20	0.60	8	15.00	200
CCRD	-1.68	10.00	0.00	0.60	-	-	-
	-1	14.00	0.08	0.84	-	-	-
	0	20.00	0.20	1.20	-	-	-
	+1	26.00	0.31	1.56	-	-	-
	+1.68	30.00	0.40	1.80	-	-	-

2.4 Estimation of oligosaccharides

The identification and quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD). A DIONEX (USA) chromatograph, supplied with a Carbopac PA1 (4x250 mm) column, a PA1 (4x50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and PEAKNET software (DIONEX , USA) were used for the analyses. Sugars were eluted with 20 mM sodium hydroxide, at a flow rate of 1.0 mL/min. Before injection, the samples were diluted with water and filtered through 0.22 μm filters (MANERA *et al*., 2010).

3. RESULTS AND DISCUSSION

For most biochemical reaction systems numerous potentially influential factors are involved, and it is not always obvious to determine which are the most important (KALIL, MAUGERI & RODRIGUES, 2000).

The first experimental design was performed in order to screen the relevant variables in GOS production. Maximum GOS yield, from the Plackett–Burman design, resulting from fermentation by *P. tsukubaensis* and *P. kluyveri* are presented in Tables 2 and 3, respectively.

Table 2: Plackett–Burman design and GOS yield produced by *P. tsukubaensi.s.*

Assay	Lactose	Yeast extract	pH	Urea	Inoculum	Agitation rate	Fermentation time (h)	Maximum GOS yield (%)[a]
1	1	-1	1	-1	-1	-1	24	17.41
2	1	1	-1	1	-1	-1	72	11.94
3	-1	1	1	-1	1	-1	72	15.77
4	1	-1	1	1	-1	1	24	18.69
5	1	1	-1	1	1	-1	48	7.96
6	1	1	1	-1	1	1	72	12.12
7	-1	1	1	1	-1	1	24	20.01
8	-1	-1	1	1	1	-1	24	19.74
9	-1	-1	-1	1	1	1	48	9.02
10	1	-1	-1	-1	1	1	24	2.17
11	-1	1	-1	-1	-1	1	24	1.21
12	-1	-1	-1	-1	-1	-1	24	1.17
13	0	0	0	0	0	0	24	4.74
14	0	0	0	0	0	0	24	5.15
15	0	0	0	0	0	0	24	4.55

[a] GOS yield (%): (g GOS/g Initial lactose)*100

Table 3: Plackett–Burman design and GOS yield produced by *P. kluyveri.*

Assay	Lactose	Yeast extract	pH	Urea	Inoculum	Agitation rate	Fermentation time (h)	Maximum GOS yield (%)[a]
1	1	-1	1	-1	-1	-1	72	12.51
2	1	1	-1	1	-1	-1	24	4.22
3	-1	1	1	-1	1	-1	24	14.47
4	1	-1	1	1	-1	1	72	14.79
5	1	1	-1	1	1	-1	24	6.10
6	1	1	1	-1	1	1	24	14.54
7	-1	1	1	1	-1	1	24	16.20
8	-1	-1	1	1	1	-1	72	16.32
9	-1	-1	-1	1	1	1	24	13.22
10	1	-1	-1	-1	1	1	72	2.11
11	-1	1	-1	-1	-1	1	48	2.68
12	-1	-1	-1	-1	-1	-1	24	2.75
13	0	0	0	0	0	0	48	7.00
14	0	0	0	0	0	0	48	8.71
15	0	0	0	0	0	0	48	8.15

[a] GOS yield (%): (g GOS/g Initial lactose)*100

For GOS produced by *P. tsukubaensis* the yield varied according to the reaction conditions, from 2.17 to 20.01%. The best results were achieved in trials 7 and 8 (Table 2). GOS produced by *P. kluyveri* also achieved the best yield of the process in trials 7 and 8 and changed from 2.11 up to 16.32% (Table 3). In assay 7 total GOS amount synthesized by *P. tsukubaensis* and *P. kluyveri* was 60.03 and 48.60 g/L, respectively.

Treichel (2009) and Rodrigues & Iemma (2005) suggest that for screening experiments p values should be set at high values, such as $p<0.10$, to make it easier to discover significant factors for later studies. Analysis of p-value (Figures 1 and 2) showed that among the tested variables only pH and urea had significant effect on GOS yield fermented by *P. tsukubaensis* and *P. kluyveri* at 90% of confidence. Although both significant independent variables positively affected the desired response, which means that a change from value -1 to +1 led to an increase in GOS yield, the pH was the more significant effect for both processes and it should be maintained at the maximum possible value.

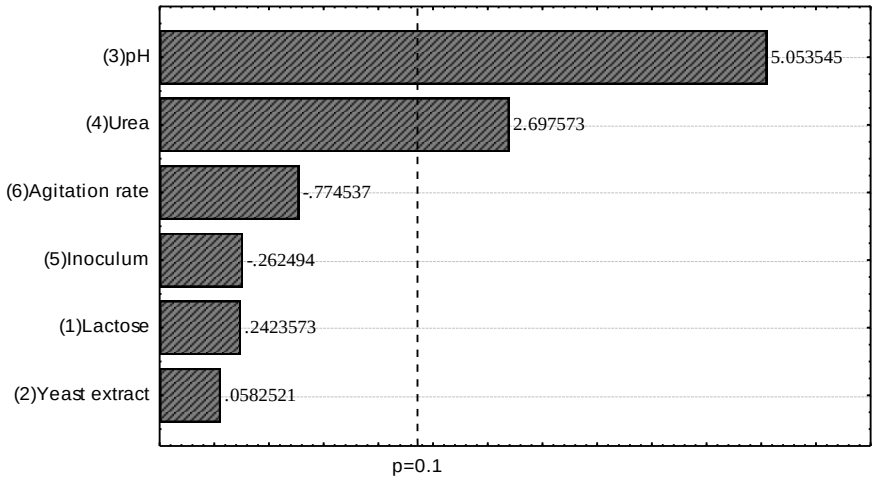

Figure 1: Standardized effect estimate (absolute value) for GOS yield produced by *Pseudozyma tsukubaensis.*

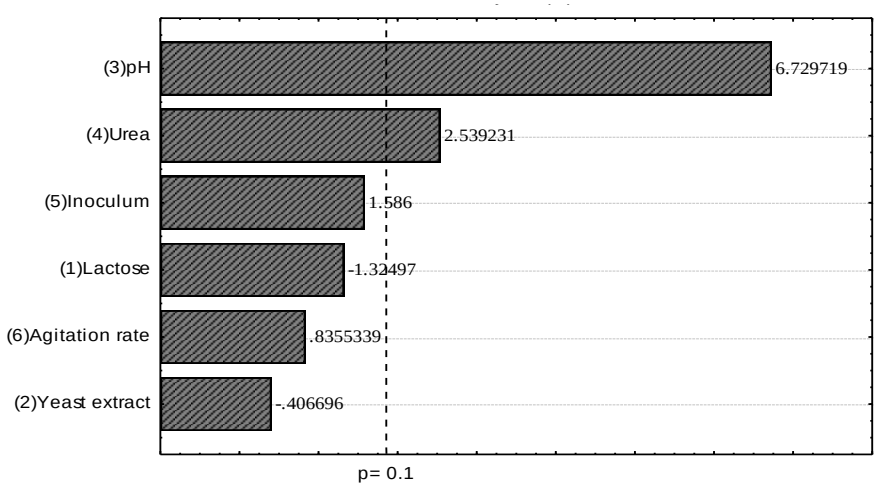

Figure 2: Standardized effect estimate (absolute value) for GOS yield produced by *Pichia kluyveri.*

As can be seen from results lactose was not significant, in the range studied, for neither of the yeasts tested. Interestingly, other empirical optimization models reported no significant effect of initial lactose concentration on GOS yield (CHEN, HSU & CHIANG, 2002; RUSTOM, FODA, & LÓPEZ-LEIVA, 1998). Goslin *et al*. (2010) suggests that given the wide acceptance that initial lactose concentration is one of the major effectors of GOS yields, these findings may point to deficiencies in these models.

It is worthy of attention that in spite of the ability for GOS synthesis by both yeast cells, the final galactose concentration in the systems was low (Table 4). This result indicates that the capacity of both yeasts strains to perform the transgalactosylation synthesis is greater than the lactose hydrolytic reaction. In addition, both yeasts demonstrated capacity to use lactose as sole carbon source at high concentrations of this disaccharide. In other words: *P. kluyveri* and *P. tsukubaensis* cells multiply in the presence of high lactose concentration and not simply stand in a non-proliferation condition while GOS are synthesized.

Table 4: Comparison of GOS and galactose production by various yeasts.

Yeast	GOS produced (g/L)	Galactose produced (g/L)	Lactose hydrolisis (%)	Reaction conditions	Reference
P. tsukubaensis	**60.03**	**11.40**	**30.00**	**Plackett-Burman assay 7**	**This study**
P.kluyveri	**48.60**	**9.00**	**24.20**		
Sirobasidium magnum	63.00	6.0	33.00	Fermentation of 30% (w/v) lactose solution, pH 7.0 at 30°C	Onishi *et al*., 1995
Sterigmatomyces elviae	74.00	5.0	30.00		
Rhodotorula minuta	67.00	7.0	40.00		

An increase of biomass in 48 hour of growth in the GOS medium was observed for both yeasts. In the first design the maximum biomass yield in assay 7 was 6.64 and 5.45 g/L for *P. kluyveri* and *P. tsukubaensis*, respectively, obtained after 48 hours.

Take into account the effect estimates concerning the first experimental design, a second one could be planned for both yeasts. However *P. tsukubaensis* fermentation responses for GOS yield presented better results, therefore this strain was selected for further optimization of the GOS production using response surface methodology.

Simply put, response surface methodology is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors and searching for optimum conditions for desirable responses (PANESAR, 2008; RODRIGUES & IEMMA, 2005).

According to the results from the Plackett-Burman design, with respect to the GOS yield obtained by *P. tsukubaensis*, lactose and yeast extract were not significant parameters, but the concentration of these compounds could be studied in a lower concentration, thus decreasing the cost of the process. Because of the pH range tolerated by

P. tsukubaensis, pH values greater than 8.0 were not studied and pH was fixed at 8.0. Inoculum concentration and agitation rate were fixed at 2.5 mL and 150 rpm, respectively, since these variables had no effect on GOS yield. Lactose, yeast extract and urea were then selected and studied using the full factorial design. Concentrations for these variables are given in Table 5.

The CCDR design was performed with data obtained at 24 hours as there was no significant increase in GOS yield after this time. Also, as the GOS yield is nearly constant, the productivity decreases gradually with fermentation time.

Table 5: Matrix of the CCRD with responses in terms of GOS yield after 24h of fermentation by *P. tsukubaensis.*

Essay	Lactose	Yest extract	Urea	GOS yield (%)[a]
1	-1	-1	-1	15.48
2	1	-1	-1	20.31
3	-1	1	-1	21.58
4	1	1	-1	19.45
5	-1	-1	1	21.98
6	1	-1	1	19.87
7	-1	1	1	24.21
8	1	1	1	20.09
9	-1.68	0	0	21.40
10	1.68	0	0	18.44
11	0	-1.68	0	20.89
12	0	1.68	0	20.95
13	0	0	-1.68	21.71
14	0	0	1.68	23.15
15	0	0	0	19.48
16	0	0	0	19.03
17	0	0	0	19.19

[a] GOS yield (%): (g GOS/g Initial lactose)*100

In this second design, GOS yield ranged from 15.48 to 24.21 %, as shown in Table 5. The best value was obtained in trial 7 with concentrations of lactose, yeast extract and urea of 14%, 0.31 and 1.56%, respectively.

The predictive Equation (1) was obtained from data in Table 5 for GOS yield with 95% of confidence.

GOS yield (%) = 19,28 – 0.62 lactose + 0.05 lactose ^{2} + 0.57 yeast extract + 0.41 yeast extract ^{2} + 0.86 urea + 0.94 urea ^{2} – 1.12 lactose. yeast extract -1.11 lactose.urea -0.3 yeast extract.urea.

The analysis of variance (ANOVA) is shown in Table 6 with 95% of confidence.

Table 6: The ANOVA for GOS yield (%) produced by *P. tsukubaensis* after 24 hours of fermentation.

Source of variation	**Sum of squares**	**Degrees of freedom**	**Means Squares**	$\mathbf{F_{cal}}$	$\mathbf{F_{tab}}$	$\mathbf{F_{cal}/F_{tab}}$
Regression	51.97	4	12.99	14.48	3.26	4.44
Residual	10.76	12	0.89			
Total	62.73	16				

For the model shown in Equation (1) F value of 14.48 (4.44 times higher than the listed F) implied that the model was significant. The model determination coefficient R^2 (0.83) suggested that the fitted model could explain 83% of the total variation, indicating that the model is predictive; therefore, it can be used for the surface generations. Response surfaces and contour plots are presented in Figure 3.

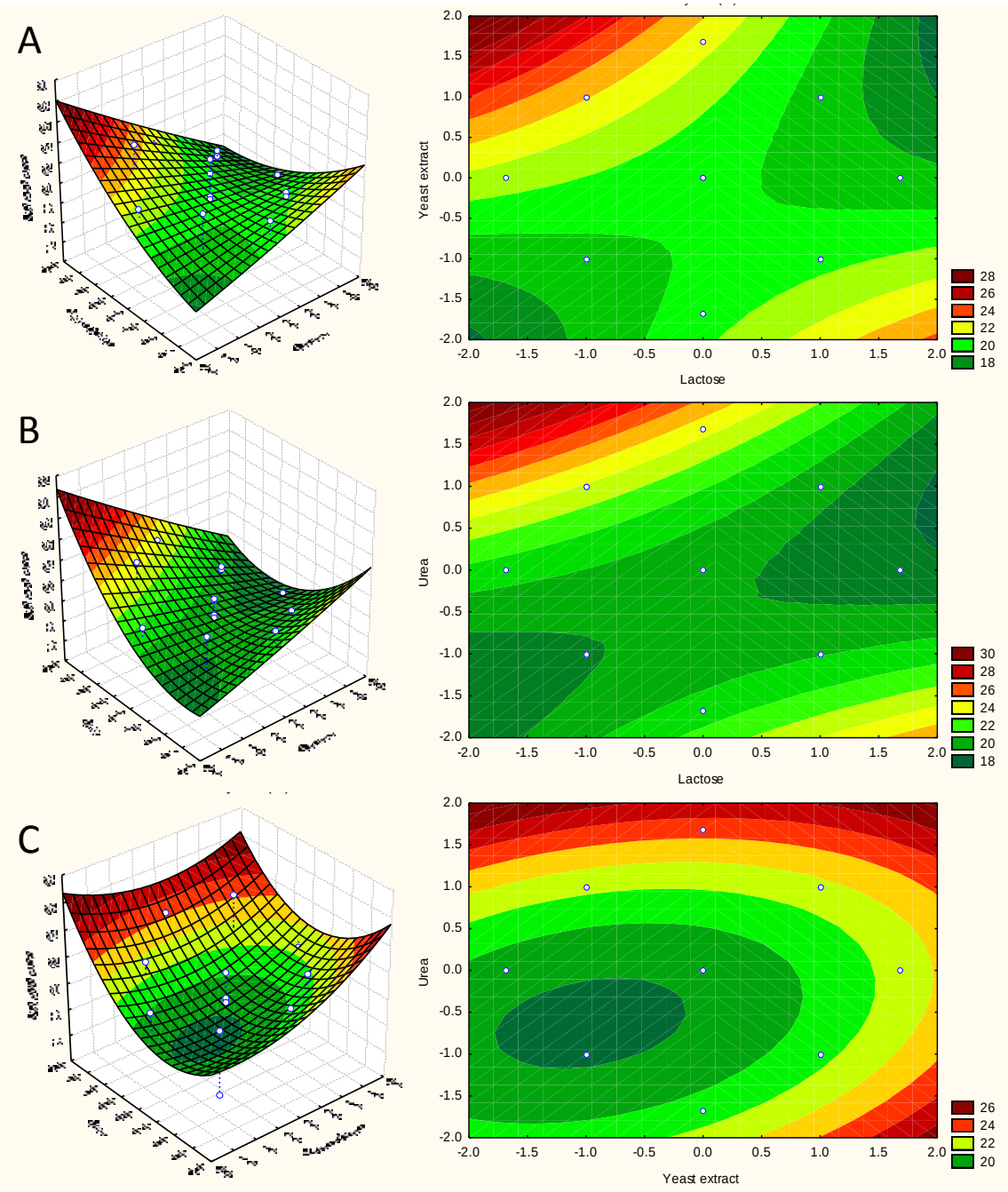

Figure 3: Response surface and contour plot for GOS yield (%) produced by *P. tsukubaensis* ~~(mN.m^{-1})~~ as a function of: lactose and yeast extract (A), lactose and urea (B) and yeast extract and urea (C)

The model was then validated, following the indications of the previous experimental design in which the uncoded values for lactose, yeast extract and urea were

26, 0.8 and 1.8%, respectively. The predicted value for GOS yield (%) under optimal conditions was 27.54%. A mean value of 28.35 % was obtained from real experiments thus proving the validity of the model. The good correlation between these results confirmed that the response model was adequate to reflect the expected optimization. Under the validated conditions the transgalactosylation activity of *P. tsukubaensis* resulted in a maximum GOS production of 73.71 g/L, with a productivity of 3.07 g/L.h and approximately 50% of lactose hydrolysis.

Manera *et al*. (2010) reported that the strategy of optimization using sequential factorial design was used to enhance GOS production using permeabilized cells of *Kluyveromyces marxianus*. The optimum conditions for GOS production were found to be: lactose concentration 500 g/L, enzyme concentration 10 U/mL, 45 °C and pH 7.0. Under optimized conditions, the GOS concentration, yield and productivity were 83 g/L, 16.5% and 27.6 g/L.h, respectively.

Rustom, Foda & López Leiva (1998) employed a two level fractional factorial experiments design to study the effects of potential factors for GOS production from whey permeate. Maximum GOS yield obtained from a 23% (v/v) lactose solution were 17.0, 22.2 and 23.5% from commercial purified β-galactosidases from *Aspergillus oryzae*, *Kluyveromyces lactis* and *K. fragilis*, respectively.

Kim, Lee & Lee (2001) observed that a maximum yield of 25.1% (w/w) GOS, which corresponds to 25.1 g/L, was obtained from the reaction of 100 g/L of lactose solution at 30°C pH 7.0 for 18 h with an intracellular crude β-galactosidase of *Kluyveromyces marxianus* var *lactis*.

4. CONCLUSION

This research demonstrated the possibility to use the yeasts *P. tsukubaensis* and *P. kluyveri* to synthesize galactooligosaccharides from lactose by fermentation. GOS producing by fermentation process can help to overcome the difficulties and expenses associated with enzyme extraction /purification from microorganism cells. It also helps to improve the development of a low-cost process for lactose hydrolysis.

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Capítulo 6

SYNTHESIS OF BIOSURFACTANT BY Pseudozyma tsukubaensis ON CASSAVA WASTEWATER AND ASSOCIATED PRODUCTION OF GALACTOOLIGOSACCHARIDES FROM LACTOSE

Resumo

Síntese de biossurfactante de *Pseudozyma tsukubaensis* em manipueira e produção associada de galactooligossacarídeos a partir da lactose.

A síntese de biossurfactante por *Pseudozyma tsukubaensis* em manipueira foi otimizada utilizando metodologia de superfície de resposta. Um processo associado de biossurfactante/biomassa para a síntese de galactooligossacarídeos a partir da lactose foi subsequentemente proposto. As condições ótimas verificadas para a produção de biossurfactante e biomassa foram de 80% (v/v) de manipueira em 200 rpm a 30°C por 48 horas. A minima tensão superficial e maxima concentração de biomassa preditas e confirmadas experimentalmente foram 26,87 mN/m e 10,5 g/L, respectivamente. A atividade de transgalactosilação obtida da biomassa da levedura, nas condições otimizadas do processo, a partir de uma solução de lactose 40% (p/v), resultou em uma produção de galactooligossacarídeos de 73,12 g/L com rendimento de 18,28% (p/p) em pH 8,0 a 30ºC por 24 horas. O biossurfactante obtido apresentou boa estabilidade em relação à temperatura (121 °C/1 h), pH (2 a 11) e força iônica (0 a 25% de NaCl), apresentando excelente atividade de emulsão, ampliando, assim, seu potencial de aplicação.

Palavras-chaves: *Pseudozyma tsukubaensis*; resíduo agroindustrial; biossurfactante; galactooligossacarídeos.

SYNTHESIS OF BIOSURFACTANT BY *Pseudozyma tsukubaensis* ON CASSAVA WASTEWATER AND ASSOCIATED PRODUCTION OF GALACTOOLIGOSACCHARIDES FROM LACTOSE

ABSTRACT

Biosurfactant synthesis by *Pseudozyma tsukubaensis* on cassava wastewater was optimized using response surface methodology and an associated process for biosurfactant/biomass for galactooligosaccharides synthesis from lactose was subsequently proposed. The optimum conditions for biosurfactant and biomass synthesis were found to be 80% (v/v) of cassava wastewater and 200 rpm at 30°C for 48 hours. The minimum surface tension and maximum biomass concentration predicted and experimentally confirmed were 26.87 mN/m and 10.5 g/L, respectively. The transgalactosylation activity of the yeast biomass at optimized conditions from 40% (w/w) lactose resulted in a galactooligosaccharides production of 73.12 g/L and a yield of 18.28% (w/w) at pH 8.0-and 30ºC in 24 hours. The biosurfactant obtained presented good thermal (121°C/1h) and pH (2 to 11) stability, tolerance to ionic strength (0 to 25% NaCl) and excellent emulsifier activity which expands its potential application.

Keywords: *Pseudozyma tsukubaensis*; agroindustrial residue; biosurfactant; galactooligosaccharides.

1. INTRODUCTION

Microorganisms synthesize a wide range of surface-active compounds, generally called biosurfactants (BANAT *et al.*, 2010). These substances possess both hydrophilic

and hydrophobic structural moieties, which in turn impart numerous unusual properties, including an ability to lower the surface tension (RUFINO, SARUBBO & CAMPOS-TAKAKI, 2007).

These polymers have attracted, in the last few years, considerable interest due to biodegradable nature, low toxicity and diversity of applications, including the food, cosmetic and pharmaceutical industries, and also environmental protection (LUNA, SARUBBO & CAMPOS-TAKAKI, 2009; MORITA *et al*., 2007a; NITSCHKE & PASTORE, 2006).

Glycolipids biosurfactants produced by yeast strains of the genus *Pseudozyma* have been stated as one of the most promising biosurfactants, due to versatile biochemical properties and high productivity from renewable resources (MOON, *et al*., 2010; FUKUOKA *et al*., 2008; KITAMOTO, ISODA & NAKAHARA, 2002).

Although biosurfactants exhibit such important advantages, a reduction in costs is an important goal to make them economically viable. The use of industrial residues with high levels of carbon sources as alternative substrates is a feasible strategy (BARROS, PONEZI & PASTORE, 2008).

Cassava wastewater (QUADROS, DUARTE & PASTORE, 2011; NITSCHKE , FERRAZ & PASTORE, 2004), waste glycerol (MORITA *et al*., 2007 b), corn steep liquor (LUNA *et al*., 2011), vegetable oil refinery residue (RUFINO, SARUBBO & CAMPOS-TAKAKI, 2007), cashew apple juice (GIRO *et al*., 2009) and potato process effluents (FOX & BALA, 2000) are examples of alternative substrates that have been suggested for biosurfactant synthesis from various microorganisms.

Cassava wastewater is a carbohydrate rich residue generated at large amounts (300 L/t of processed roots) during the production of cassava flour, a common ingredient used

on Brazilian cookery (NITSCHKE & PASTORE, 2006). The disposal of cassava wastewater causes environmental problems owing to its high organic load, although this waste is composed of carbohydrates, nitrogen, minerals, and trace elements and therefore it is a very attractive substrate for biotechnological processes (NITSCHKE & PASTORE, 2003).

The use of cassava wastewater as low cost substrate for biosurfactant and biomass production by *Pseudozyma tsukubaensis* in submerged fermentation was optimized. It was also proposed an interesting possibility of using an aliquot of the cell suspension grown on cassava wastewater to be used as the inoculum to synthesize galactooligosaccharides from lactose by fermentation.

2. MATERIALS AND METHODS

2.1 Microorganism

The yeast strain used in this study was previously isolated from health nectarine (*Prunus persica* var. *nucipersica*) and identified by 16S-rRNA cDNA sequence as *Pseudozyma tsukubaensis* (not yet published).

2.2 Preparation of the substrate

Cassava wastewater was collected from a cassava flour factory (Plaza LTDA, Santa Maria da Serra, SP, Brazil) and transported to the place of processing at room temperature. It was homogenized, boiled, cooled, centrifuged at 10.000 rpm for 20 min and stored at -18º C until used (BARROS, PONEZI & PASTORE, 2008).

2.3 Optimization of biosurfactant production

All experiments were carried out in Erlenmeyer flasks containing cassava wastewater in various concentrations, according to the experimental design (Table 1). The media were sterilized by autoclaving at 121 °C for 20 min. The inoculum of *P. tsukubaensis* was prepared by transferring cells grown on a slant to YEPD broth (medium YEPD). The seed culture was maintained at 30°C for 48 h in a rotary shaker incubator at a speed of 150 rpm. Each flask containing cassava wastewater was inoculated with 10% (v/v) seed culture. The inoculum was standardized by measuring the optical density at λ= 600 nm. Thus, considering the standard curve for cell concentration versus optical density, a volume of inoculum sufficient for the culture to reach the initial concentration of $1x10^7$ CFU/mL was added to the medium. The flasks were incubated for 48 h at 30 °C in a rotatory shaker at 150 rpm. At regular intervals, an aliquot of the reaction mixture was removed for surface tension, pH and absorbance at 600 nm determinations.

In order to find the optimal conditions for biosurfactant synthesis, a central composite rotational design CCRD for two independent variables with three replicates at the central point and four axial points (11 trials) was performed (RODRIGUES & IEMMA, 2005). The independent variables were cassava wastewater concentration (30-100% v/v) and agitation rate (0-300 rpm). The software Statistica 7.0 (Statsoft Inc., USA) was used to analyze the results.

2.4 Determination of superficial tension

To evaluate biosurfactant production, pre-inoculum was centrifuged at 10.000 rpm for 10 minutes and the supernatant used to determine superficial tension [TS], critical micelar dilution 1/10 [CMD^{-1}] and 1/100 [CMD^{-2}] with a Krüss GmbH K-12 processor

tensiometer (Hamburg, Germany) using the Wilhelmy plate method (NITSCHKE & PASTORE, 2006).

2.5 Growth measurement

Cellular growth was measured by optical density of the culture at 600 nm and biomass concentrations (g dry weight/L) were determined using a calibration curve. The calibration curve was calculated using dilutions of a biomass suspension thus, a relationship between biomass concentration (g/L) and optical density (600 nm) was determined.

2.6 Biosurfactant characterization

To characterize the biosurfactant produced by this strain of *Pseudozyma tsukubaensis*, studies of stability and emulsification were performed. In order to obtain the large quantity of biosurfactant necessary for these experiments, they were carried out using a cell-free foam obtained by fermentation in bioreactor using cassava wastewater as substrate, at 30 °C, 150 rpm and 4 vvm of aeration.

2.6.1 Stability studies

To evaluate the biosurfactant stability to heat, samples were heated in a boiling water bath and in an autoclave at 121 °C for different time intervals and cooled at room temperature. The pH stability was accomplished by adjusting the broth to different pH values from 2 to 12 with NaOH or HCl 1N and 0.1N as needed. To study the effect of salt addition on biosurfactant, 1 mL of the cell-free foam was added to test tubes containing 9 mL of distilled water and a stock solution of NaCl in a proportion necessary to reach concentrations from 2 to 25% of the salt. The surface tension and CMD values of each

treatment were assayed as described above (BARROS, QUADRO & PASTORE, 2008; NITSCHKE & PASTORE, 2006).

2.6.2 Emulsification index

The emulsification index was measured using the method described by Cooper and Goldenberg (1987) whereby 6 mL of hydrocarbon was added to 4 mL of the cell-free broth in a screwcap test tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h, and the emulsification index (E24) was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100. Sodium dodecyl sulphate (SDS) was used for emulsification index comparison.

A mineral motor oil (Lubrax Essencial Petrobrás – SF SAE 40) was tested, as well as the vegetable oils from: canola (Salada - Bunge), sunflower (Salada - Bunge), corn (Salada - Bunge), sunflower with Brazil nut (Salada – Bunge), palm (Cepêra) and soybean (Lisa - Cargill).

2.7 Synthesis of galactooligosaccharides (GOS)

A cell suspension (2.5 mL) of the fermented cassava wastewater was inoculated into 50 mL of a medium comprising 40% lactose (w/v), 0.5% of yeast extract (w/v), 0.1% (w/v) of urea in 200 mM potassium phosphate buffer (pH 8.0) and cultivated aerobically at 30°C for 24 h in a reciprocal shaker at 150 rpm. The reaction was stopped in boiling water bath for 10 min to inactivate the enzyme, then diluted, and filtered through a 0.45 µm membrane to remove insoluble particles. Oligosaccharides formed were analyzed by described in 2.8.

2.8 Estimation of oligosaccharides

The identification and quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD). A DIONEX (USA) chromatograph, supplied with a Carbopac PA1 (4x250 mm) column, a PA1 (4x50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and PEAKNET software (DIONEX , USA) were used for the analyses. Sugars were eluted with 20 mM sodium hydroxide, at a flow rate of 1.0 mL/min. Before injection, the samples were diluted with water and filtered through 0.22 μm filters (MANERA *et al*., 2010).

3. RESULT AND DISCUSSION

Optimization of biosurfactant production

This paper describes the use of cassava wastewater as low cost medium for biosurfactant production by *Pseudozyma tsukubaensis*. This synthesis was detected by surface tension reduction when the microorganism was cultivated on cassava wastewater, in various concentrations using different stirring conditions according to the factorial design (Table 1). .

As can be seen in Table 1 superficial tension ranged from 26.30 to 48.39 mN/m. Biomass concentration changed from 3.44 up to 11.55 g/L.

Results demonstrated a direct relation between biosurfactant production (shown by a decrease in the surface tension) and biomass concentration. Similar behavior was reported by Rodrigues *et al*. (2006) using response surface methodology in order to optimize the

medium for biosurfactants production by probiotic bacteria *Lactococcus lactis* and *Streptococcus thermophilus*.

Although previous works showed that cassava wastewater is an interesting substrate for biosurfactant production by *Bacillus* species (QUADROS, DUARTE & PASTORE, 2011; BARROS, PONEZI & PASTORE, 2008; NITSCHKE & PASTORE, 2003), as far as we know this study is the first report of biosurfactant synthesis by yeast using this agroindustrial waste as an alternative medium.

In this study cassava wastewater proved to be a suitable alternative substrate for *P. tsukubaensis* growth and biosurfactant production. The ability of the strain studied to reduce surface tension was in agreement with results obtained by others for biosurfactants production by *Pseudozyma* species in which surface tension values vary from 25.1 to 30.7 mN/m (KONISHI et al., 2008; MORITA et al., 2008 a; MORITA *et al*., 2008 b).

Table 1: Matrix of the CCRD (coded and real and values) with responses in terms of superficial tension (N/m) and biomass (g/L) after 48h of fermentation.

Essay	Cassava wastewater concentration % (v/v)	Agitation (rpm)	Superficial tension (mN/m)	Biomass concentration (g/L)
1	-1 (40.18)	-1 (43.62)	43.78	3.44
2	+1 (89.82)	-1 (43.62)	38.67	7.89
3	-1 (40.18)	+1 (256.4)	35.18	5.46
4	+1 (89.82)	+1 (256.4)	27.1	11.55
5	-1,41 (30)	0 (150.00)	43.02	4.90
6	+1,41 (100.00)	0 (150.00)	27.52	10.18
7	0 (65.00)	-1,41 (0.00)	48.39	2.81
8	0 (65.00)	+1,41 (300.00)	28.32	9.54
9	0 (65.00)	0 (150.00)	27.42	10.4
10	0 (65.00)	0 (150.00)	26.96	9.00
11	0 (65.00)	0 (150.00)	26.30	10.09

It is noteworthy that the pH of cassava wastewater was not adjusted for optimization tests and at central points it was observed a slight increase in pH from 5.5 to 6.7 ±0.19 after 48 hours of fermentation.

Neglecting the non-significant terms, the predictive Equations (1) and (2) were obtained from data in Table 1, for superficial tension and biomass concentration, respectively, with 95% of confidence.

Superficial tension (mN/m) = 26.89 - 4.38 cassava + 4.03 cassava ^{2} - 6.06 agitation + 5.57 agitation ^{2} (1)

Biomass concentration (g/L) = 9.83 + 2.25 cassava – 1.08 cassava ^{2} + 1.89 agitation – 1.77 agitation ^{2} (2)

The analysis of variance (ANOVA) is shown in Tables (2) and (3) for superficial tension and biomass concentration, respectively, with 95% of confidence. For the model shown in Equation (1) F value of 45.81 (10.11 times higher than the listed F) implied that the model was significant. The model determination coefficient R^2 (0.96) suggested that the fitted model could explain 96% of the total variation. In the case of the model presented in Equation (2), F ratio was 6.04 times higher than its critical value and R^2 was 0.94. This data indicates that both models are predictive; therefore, they can be used for the surface generations.

Table 2: The ANOVA for superficial tension (mN/m)

Source of variation	Sum of squares	Degrees of freedom	Means squares	F_{cal}	F_{tab}	F_{cal}/F_{tab}
Regression	659.63	4	164.88	45.81	4.53	10.11
Residual	21.59	6	3.59			
Total	681.12	10	68.11			

Table 3: The ANOVA for biomass (g/L)

Source of variationo	Sum of squares	Degrees of freedom	Means squares	F_{cal}	F_{tab}	F_{cal}/F_{tab}
Regression	89.09	4	22.27	27.39	4.53	6.04
Residual	4.87	6	0.81			
Total	93.97	10	9.39			

Response surface and contour plot for the superficial tension and biomass concentration are presented in Figure 1 and 2, respectively.

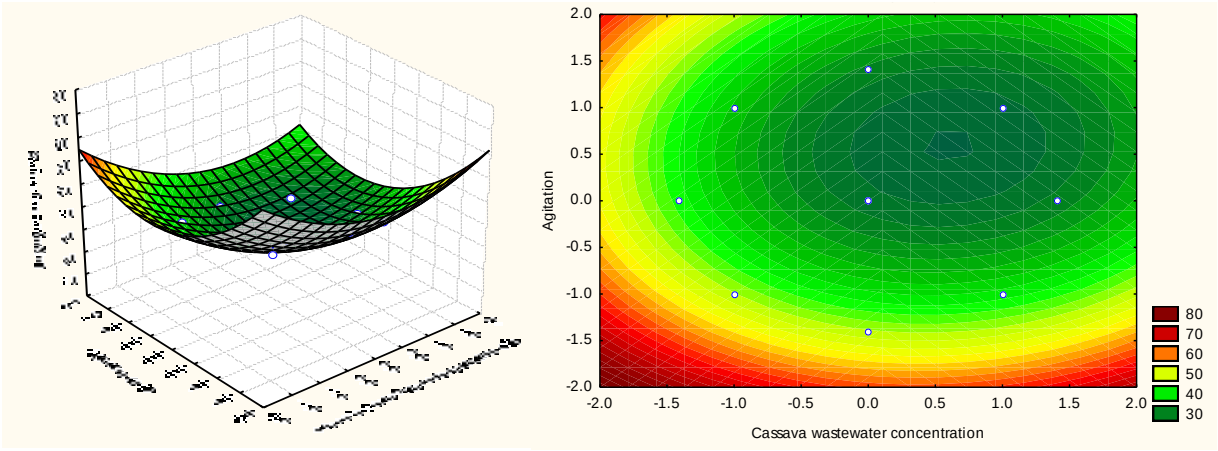

Figure 1: Response surface and contour plot for biosurfactant production by *P. tsukubaensis* ($mN.m^{-1}$) as a function of cassava wastewater concentration and agitation speed.

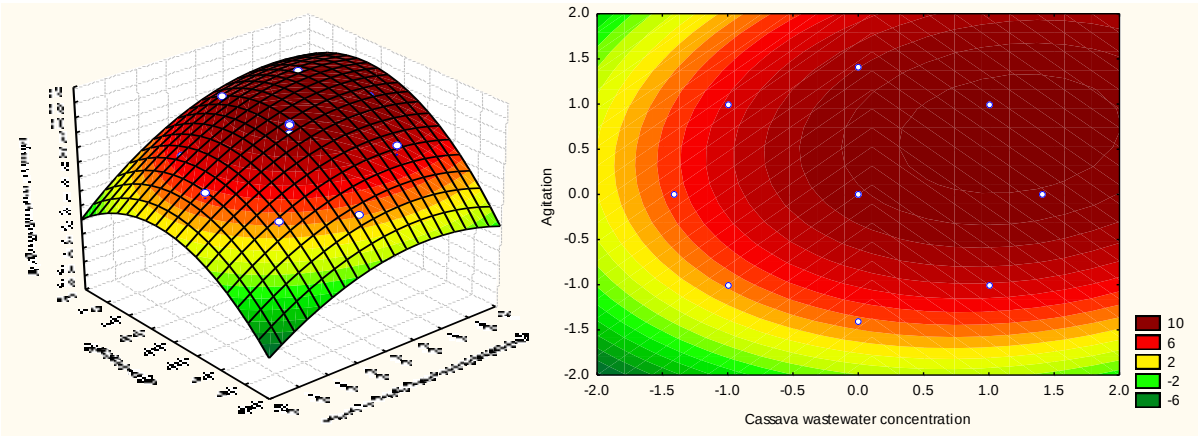

Figure 2: Response surface and contour plot for biomass production by *P. tsukubaensis* ($g.L^{-1}$) as a function of cassava wastewater concentration and agitation speed.

The corresponding optimum uncoded values for cassava wastewater and rotation were 80% (v/v) and 200 rpm, respectively. Under optimal conditions, the model predicted a response of 25.00 mN/m for superficial tension and 11.18 g/L for biomass.

In order to validate the predicted results of the model, experiments using the deduced optimal condition were carried out in triplicate.

A mean value of 26.87 ± 0.31mN/m and 10.5 ± 0.55g/L for superficial tension and biomass, respectively, were obtained from real experiments thus proving the validity of the model. The good correlation between these results confirmed that the response model was adequate to reflect the expected optimization.

Luna *et al*. (2011) employed statistical experimental designs to improve the tension-active emulsifying agent produced by *Candida sphaerica* using a low cost substrate formulated with soybean oil refinery residue and corn steep liquor. The experiments showed that the lowest surface tension was 25.25 mN/m in the trial formulated with 9% (v/v) of each residue.

The strategy of optimization using sequential factorial design was used to enhance the tensio-active emulsifying agent produced by *Candida lipolytica* using soybean oil refinery residue as substrate. Reduction in the surface tension, which indicates the presence of biosurfactant, reached a value of 25.29 mN/m (RUFINO *et al*., 2008).

Synthesis of GOS

The strain *P. tsukubaensis* has already been described for GOS production from lactose by fermentation (not yet published). GOS, containing 3–10 molecules of galactose and glucose, are principally synthesized by enzymatic transgalactosylation reaction from lactose by β-galactosidases from various microorganisms and can be produced either by isolated enzymes or by fermentation (GOSLIN *et al*., 2010).

Because of their safety, stability, organoleptic properties, resistance to digestion in the upper bowel and fermentability in the colon, as well as their abilities to promote the growth of beneficial bacteria in the gut, these prebiotics are being increasingly incorporated into diet (MACFARLANE, STEED & MACFARLANE, 2007).

In this study integrated process for biosurfactant and GOS synthesis was proposed. In this way, in order to integrate both processes, it was necessary to prove that *P. tsukubaensis* cultivated for 48 h in the cassava wastewater medium for biosurfactant production was still active to be used as the inoculum for GOS synthesis from lactose by fermentation.

In the proposed associated production process a cell suspension of the fermented cassava wastewater at the optimum conditions validated was inoculated into a 40% (w/w) lactose solution. The transgalactosylation activity of *P. tsukubaensis* resulted in a maximum GOS production of 73.12 ± 0.339 g/L, with a productivity of 3.04 ± 0.014 g/L.h and a yield

of 18.28 ± 0.084%. Thus, it was proved that the biomass resulting from the biosurfactant process has kept the capacity to ferment lactose to produce GOS.

Similar results for GOS production were found by Onishi, Yamashiro & Yokozeki (1995) screening numerous microorganisms for the ability to produce this prebiotic. Those authors found that total GOS synthesized by *Sirobasidium magnum*, *Sterigmatomyces elviae* and *Rhodotorula minuta* was 67.73 and 74 g/L from 30% (w/w) in 16 h at 30°C. Onish & Tanaka (1998) using a recycling cell culture of *Sterigmatomyces elviae* to produce GOS maintained at high levels during six cycles of production, with the amount of GOS produced in each cycle being more than 216 g/L from 40% (w/w) lactose in 68 h, with a productivity of 3.17 g/L.h. Among 47 strains of yeasts isolated from Korean traditional *Meju Kluyveromyces maxianus* var *lactis* showed the best ability to produce GOS, reaching a maximum yield of 25.1% (w/w) GOS, which corresponds to 25.1 g of GOS/L, from 10% (w/w) lactose solution at 30 °C in 18 h (Kim, Lee & Lee, 2001).

Biosurfactant properties

The stability of the surface tension is an important factor for the utilization of biosurfactants under specific environmental conditions (RUFINO *et al*., 2008).

Studies on the effect of heat treatment (100 °C) demonstrated that no appreciable change in the surface tension of the cell-free foam produced by *P. tsukubaensis* in the medium had occurred (Figure 3).

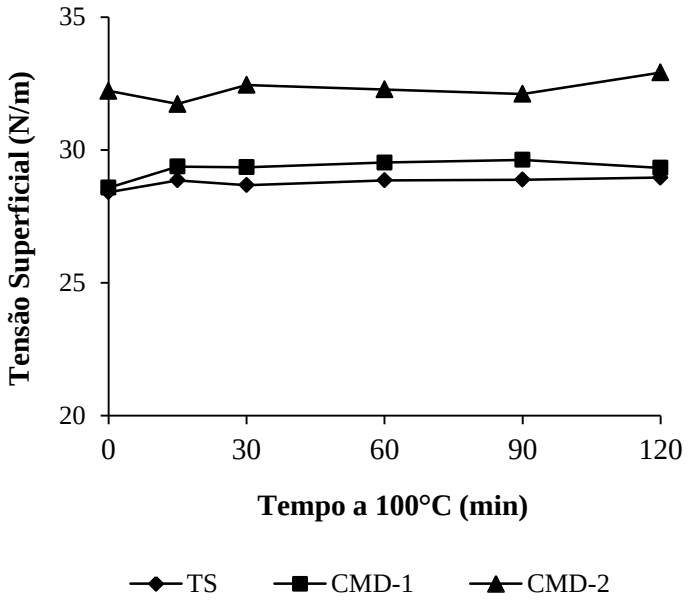

Figure 3: Heat (100°C) stability of the biosurfactant from *P. tsukubaensis* (ST: surface tension; CMD^{-1}, CMD^{-2}: critical micelle dilutions).

The results obtained showed that the biosurfactant was thermally stable even when submitted to autoclave sterilization (121 °C) for 1 hour (Figure 4).

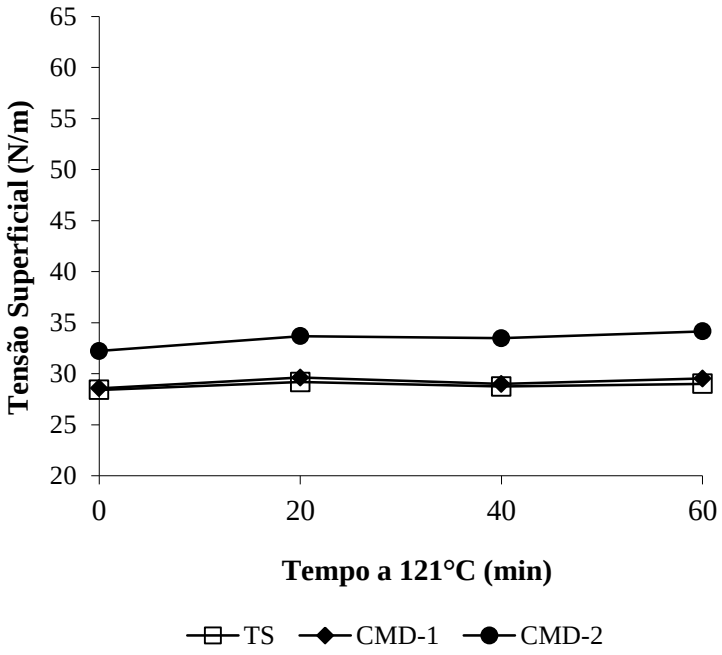

Figure 4: Heat (121 °C) stability of the biosurfactant from *P. tsukubaensis* (ST: surface tension; CMD^{-1}, CMD^{-2}: critical micelle dilutions).

As can be seen in Figure 5, the surface tensions and CMD^{-1} were maintained over a pH range of 2–11 with minimal deviation, whereas CMD^{-2} shown a gradual increase on surface tension with increasing pH values. The change detected at pH 12 must be a result of proteinaceous components denaturation or increased ionization under extremes of pH, according to Ghurye, Vipulanandan & Willson (1994).

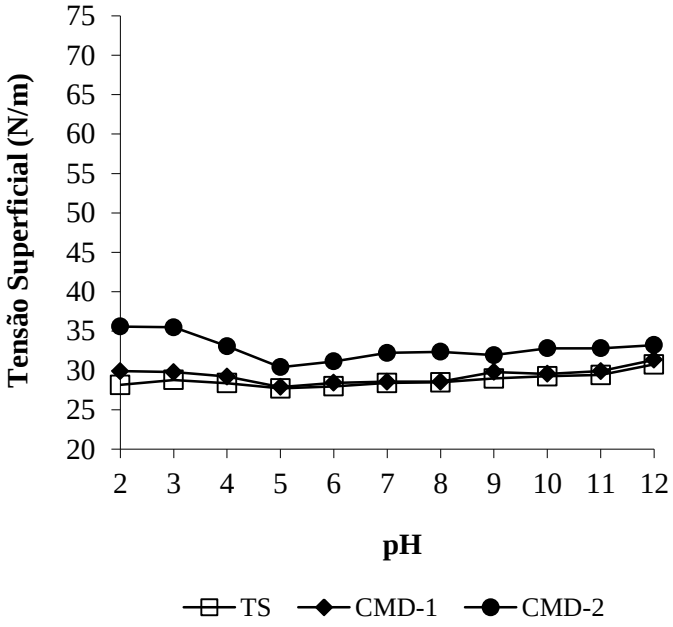

Figure 5: Influence of the pH on the biosurfactant from *P. tsukubaensis* surface activity (ST: surface tension; CMD^{-1}, CMD^{-2}: critical micelle dilutions).

In order to verify the feasibility of using the biosurfactant produced in high salt environments, like marine environment or some industrial wastewater, the resistance to salts was evaluated. In order to minimize weighting errors, 9 mL of the dilutions of a stock solution of NaCl had to be added to 1 mL of the biosurfactant solution, so that it was not possible the ST measurement. However, the lack of effect of sodium chloride addition on surface tension can be easily observed only with the CMD values (Figure 6).

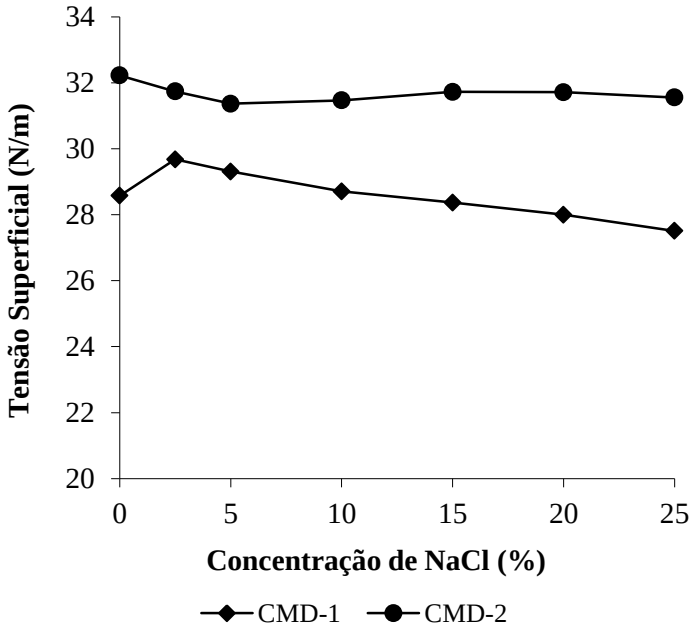

Figure 6: Effect of the salt concentration on the biosurfactant from *P. tsukubaensis* surface activity (ST: surface tension; CMD^{-1}, CMD^{-2}: critical micelle dilutions).

In addition to surface and interfacial tension, stabilization of an oil and water emulsion is commonly used as a surface activity indicator (RUFINO, SARUBBO & CAMPOS-TAKAKI, 2007). Results on the emulsification activities of the biosurfactant obtained from *P. tsukubaensis* with various water-immiscible substrates are presented in Table 4. Even though emulsification activities for the biosurfactant were lower than those found for Sodium dodecyl sulphate, with the exception of E_{24} for the palm oil, the comparison of its E_{24} and E_{120} values showed that product was capable of forming stable water-in-oil emulsions with all oils tested. These findings suggest that the biosurfactant synthesized in this study is a good candidate as a cleaning and emulsifying agent in and it has potential applications in microbial enhanced oil recovery, environment preservation, medicine food and cosmetics industry (GONG *et al*., 2009).

Table 4: Percentage of emulsification activity after 24 (E_{24}) and 120 hours (E_{120}) of the biosurfactant and SDS against vegetal and mineral oils.

Oil	Biosurfactant		SDS	
	E_{24}	E_{120}	E_{24}	E_{120}
Soy	51%	55%	70%	71%
Canola	52%	42%	77%	68%
Sunflower	58%	60%	75%	73%
Corn	57%	43%	78%	76%
Brazil nut	51%	61%	78%	74%
Palm	36%	15%	17%	17%
Motor (mineral oil)	64%	60%	78%	77%

4. CONCLUSION

All the characteristics described above demonstrate the potential use of cassava wastewater as an alternative substrate for biosurfactant production by *P. tsukubaensis*, providing biomass growth and product accumulation.

The produced biosurfactant exhibited interesting and useful properties such as: surface tension reduction, stability at high temperatures and pH extremes and tolerance to ionic strength. It also presented ability to form emulsions with vegetal and mineral oils and once it is synthesized in a residue may be produced on a large scale from low-cost for commercial applications.

This research also demonstrated the possibility to use the yeast *P. tsukubaensis* to synthesize galactooligosaccharides from lactose with promising results in an associated process with biosurfactant obtainment.

Additional studies are being conducted to characterize the biosurfactant produced in this study and to scale up this process. This in turn can provide a focus for effective use of

P. tsukubaensis to synthesize two economical biotechnological products of increase interest.

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CONCLUSÕES GERAIS

O processo de imobilização covalente em celite de β-galactosidase comercial de *Aspergillus oryzae* (Sigma®), se mostrou eficiente para a produção de GOS e principalmente para a hidrólise da lactose. Esta mesma enzima imobilizada covalentemente em quitosana, apresentou uma alta produtividade de formação de GOS logo no início do processo e no final da reação a hidrólise da lactose também foi eficiente.

Em ambos os casos, os derivados imobilizados apresentaram robustez frente a condições adversas de pH e temperatura quando comparados à β-galactosidase livre e boa estabilidade operacional. A vida útil da enzima imobilizada em celite foi bastante superior à imobilizada em quitosana, fato este expectável, uma vez que este último é um composto orgânico (polissacarídeo) e, assim sendo, é mais susceptível à contaminação microbiana durante o armazenamento.

As leveduras endofíticas *Pseudozyma tsukubaensis* e *Pichia kluyveri* se revelaram hábeis produtoras de GOS, a partir da fermentação da lactose, apresentando forte atividade de transgalactosilação e baixa capacidade de hidrólise da lactose. Não obstante esta constatação, a otimização estatística deste processo foi capaz não só de aumentar o rendimento de GOS, bem como de reduzir a concentração de lactose final no sistema.

Este foi o primeiro estudo a demonstrar a capacidade de síntese destes oligossacarídeos por estas leveduras, sendo que a cepa de *P. kluyveri* também se revelou um potencial probiótico frente a ensaios preliminares *in vitro*.

A cepa de *P. tsukubaensis*, por sua vez, foi capaz de sintetizar biossurfactante em manipueira, um resíduo agroindustrial da indústria de farinha de mandioca. Dentro do nosso conhecimento, este foi o primeiro relato da produção deste composto por este gênero microbiano em um substrato altamemente polar e, além do mais, de baixo custo. O biossurfactante obtido mostrou uma excelente capacidade de reduzir a tensão superficial e de emulsificação de óleos diversos, sendo ainda bastante estável frente à condições adversas de pH, temperatura e força iônica.

Foi também a primeira pesquisa a descrever a produção associada de biossurfactante e GOS, sendo o processo desenvolvido neste trabalho bastante interessante, economicamente viável e ambientalmente correto.

Por fim, ao comparar os diferentes processos de síntese de GOS empregados neste estudo, pode-se dizer que a hidrólise da lactose foi mais efetiva nos sistemas de β-galactosidase de *A. oryzae* (Sigma®) imobilizada, sendo a produtividade de formação de GOS também maior. Por outro lado, o processo de obtenção de GOS utilizando as leveduras isoladas, é mais simples (não requer etapa de isolamento e purificação da enzima) e de menor custo. Neste processo também se evidenciou maior rendimento de GOS após a otimização utilizando a metodologia de superfície de resposta.

Como sugestão para trabalhos futuros, visando aperfeiçoar e dar continuidade a esta pesquisa, é interessante que se investiguem alguns pontos, tais como:

- Estudar a produção de GOS por *P. kluyveri* e *P. tsukubaensis* no meio de cultura otimizado, porém, elevando a temperatura e diminuindo o tempo de reação;

- Realizar testes de fermentabilidade e digestibilidade dos GOS produzidos neste estudo;

- Testar a capacidade de fermentabilidade da cepa de *P. kluyvery* no GOS produzido por esta mesma levedura, visando a formação de um interessante simbiótico;

- Investigar as propriedades biológicas dos GOS sintetizados pelas leveduras isoladas neste trabalho, através de ensaios *in vitro* e *in vivo*;

- Complementar o estudo do potencial probiótico da cepa de *P. kluyveri*, isolada nesta pesquisa, através de ensaios *in vivo*;

- Caracterizar o biossurfactante sintetizado pela levedura *P. tsukubaensis*;

- Avaliar a produção do biossurfactante de *P. tsukubaensis* em outros substratos alternativos e estudar algumas de suas possíveis aplicações.